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14. ABSTRACT

This research project had three specific aims. The first was to determine which tissue in the rat mammary gland was the target of the chemical carcinogen N-nitrosomethylurea. This aim was fulfilled. The second aim included screening and counting lesions and performing morphometric data analysis of whole mounts (branching pattern, relative abundance of the different ductal and alveolar structures). These studies aimed at identifying the changes occurring between the time of exposure and the appearance of neoplasias. This aim was also fulfilled. Finally, as described for Aim #3, during years two and three we explored the specific roles of hyaluronan and emmprin, two molecules that are enriched in tumors and involved in tumorstromal cell interactions as mediators of neoplastic initiation and progression.

15. SUBJECT TERMS

Carcinogenesis, tissue transplants, nitrosomethylurea, pathology, immunohistochemistry, EMPRIN

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INTRODUCTION:

This research project had three specific aims. The first was to determine which tissue in the rat mammary gland was the target of the chemical carcinogen N-nitrosomethylurea. This aim was fulfilled. The second aim included screening and counting lesions and performing morphometric data analysis of whole mounts (branching pattern, relative abundance of the different ductal and alveolar structures). These studies aimed at identifying the changes occurring between the time of exposure and the appearance of neoplasias. This aim was also fulfilled. Finally, as described for Aim #3, during years two and three we explored the specific roles of hyaluronan and emmprin, two molecules that are enriched in tumors and involved in tumor-stromal cell interactions as mediators of neoplastic initiation and progression.

BODY:

HYPOTHESIS: Are the targets of the carcinogen the genomic DNA of epithelial cells, the stroma, or both? We satisfactorily answered this question and we have accomplished the following:

Aim #1 was completed. The results were published (Maffini MV, Soto AM, Calabro JM, Ucci AA, Sonnenschein C. The stroma as a crucial target of chemical carcinogens in the rat mammary gland. Journal of Cell Science, 117:1495-1502, 2004). Details are therein.

We observed that only those animals whose stroma was exposed to NMU developed neoplasias, regardless of whether or not the transplanted mammary epithelial cells were exposed to the carcinogen. The Ha-ras mutation was also assessed in DNA isolated from NMU-exposed and non-exposed mammary epithelial cells, mammary fibroblasts, and mammary pre-adipocytes collected from intact virgin rats and grown in vitro. The presence of the mutation did not correlate with cell type, culture conditions or carcinogen treatment.

These results highlighted the need to explore the roles that the stroma components, i.e. the cells (fibroblasts, adipocytes, macrophages, mast cells) and the extracellular matrix, play in rodent mammary carcinogenesis. Efforts are now being directed at exploring the role of the stroma in 3-dimensional tissue culture models for carcinogenesis involving novel silkworm fibroins that form scaffoldings and mats on which cellular components of the mammary gland will be grown.

Aim #2. To establish a pattern of the dynamic response of the stroma and the epithelium of the different combinations of tissue involved in mammary carcinogenesis as outlined in **Aim #1**.

As stated in the FIRST YEAR REPORT we observed that documenting the time-course of cell repopulation of the cleared mammary gland could not be accomplished as proposed, because it took at least 30 days for the formation of a noticeable ductal tree. By 150 days, 1/3 of the "cleared" fat pad was covered with the tree-like epithelial growth. We decided, instead, to explore a related phenomenon that will shed light on the role of the stroma in carcinogenesis. Given that mammary glands are most vulnerable to chemical carcinogenesis at puberty and become resistant as the animal ages, we asked the question: Are these properties due to changes in the stroma? We assessed whether the ability of the cleared mammary gland fat pad (CFP) to normalize cancer cells varies in diverse physiological states, for example, the age of the host. This pilot study revealed that epithelial carcinoma cells (ECCs) formed tumors when injected in CFPs of "young" (24 and 50 day-old) hosts. In contrast, tumor formation was substantially decreased or absent when tumor cells were injected into CFP of "adult" (80 and 150 day-old) or multiparous hosts (after 2 pregnancies). Most remarkably, these data suggest a parallel to the phenomenon of age-dependent susceptibility and resistance to chemical carcinogens. This experiment was completed during the third year of funding and is now in press in the American Journal of Pathology (on line version by November 1, 2005)
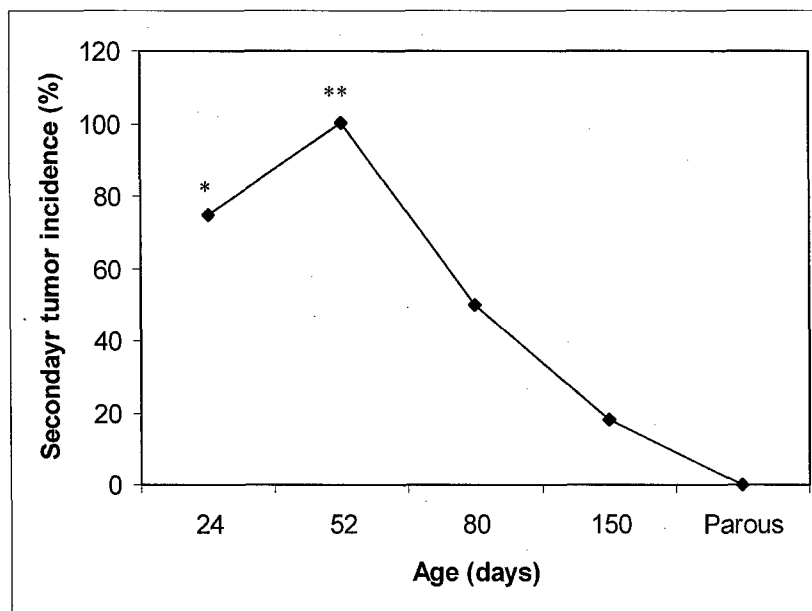
Fig.1: The incidence of secondary tumors decreased with the age of the stroma. The parous host only developed normal ductal outgrowths. **Statistically different from twice-parous, 150- and 80-day-old host groups. * Statistically different from twice-parous, and 150-day-old host groups.

Hence, susceptibility to chemical carcinogens and the ability to reprogram the neoplastic behavior seem to be linked to aging, more specifically, the ability of these tumors cells to proliferate is regulated by the age of the stroma in which they are implanted. That is, as susceptibility to carcinogenesis decreases, the ability of the stroma to reprogram neoplastic epithelial cells increases. This observation strongly supports the notion that the neoplastic phenotype is context-dependent and, hence, it offers the intriguing possibility that the process of carcinogenesis is amenable to normalization or "cure" once the mechanisms of stroma-mediated "normalization" are elucidated.

Aim #3. To define the relationship between early carcinogenic events and peri- and extracellular markers that are known to affect the proliferative and invasive behavior of cancer cells. We have optimized the protocols to characterize EMMPRIN and hyaluronic acid expression using immunohistochemistry and histochemistry techniques, respectively. Quantification of hyaluronic acid using an ELISA assay could not be performed due to technical problems. We are starting to test the specific roles of hyaluronan and emmprin, two molecules that are enriched in tumors and involved in tumor-stromal cell interactions, as mediators of neoplastic initiation and progression.

To accommodate a novel perspective on the role of the stroma in carcinogenesis, a rigorous analysis of concepts, definitions and experimental approaches is now needed. This will facilitate the identification of the mediators responsible for the altered tissue phenotype in cancers and of ways to reverse their effect by adopting a solid epigenetic perspective.

KEY RESEARCH ACCOMPLISHMENTS:
• We have completed **Aim 1**: the results clearly establish that the stroma is a main target of chemical carcinogens and suggest that carcinogenesis is a tissue organization-based problem.
• Our data collected while developing **Aim 2** strengthened the notion that the stroma is the most prominent target of the carcinogen and, equally important, the stroma has the capacity to "normalize" the neoplastic properties of rat mammary gland tumors.
• The histochemical techniques needed to explore **Aim # 3** have been optimized. We are currently analyzing the data.

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REPORTABLE OUTCOMES:
• Preliminary data was presented in the 12th International Conference of the International Society of Differentiation. (see enclosed abstract) A paper was published reporting the results of **Aim # 1**: (see enclosed JCS paper).
• Presentations of our observations were made at the 2002 and 2003 Gordon Research Conferences, the 2005 Keystone Conferences and 2005 DOD Era of Hope meetings.

CONCLUSIONS:
Our theory–neutral experimental design tested whether the primary target of the carcinogen was the epithelium, the stroma, or both tissue compartments. We observed that neoplastic transformation of mammary epithelial cells occurred only when the stroma was exposed *in vivo* to N-nitrosomethylurea, regardless of whether or not the epithelial cells were exposed to the carcinogen. Mutation in the Ha-ras-1 gene did not correlate with initiation of neoplasia. Our results suggest that the stroma is a crucial target of the carcinogen and that mutation in the Ha-ras-1 gene is neither necessary nor sufficient for tumor initiation.
Further, we have found evidence that the ability of the stroma to induce and to curtail neoplastic behavior is age-dependent. At the same time, we observed that the neoplastic properties of rat mammary gland tumor cells can be restrained and "normalized" so that they could form normal ductal structures.

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Maffini MV, Soto AM, Calabro JM, Ucci AA, Sonnenschein C. The stroma as a crucial target of chemical carcinogens in the rat mammary gland. Journal of Cell Science 117:1495-1502, 2004.

Soto AM & Sonnenschein C. The somatic mutation theory of cancer: growing problems with the paradigm? BioEssays 26:1097-1107, 2004.

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APPENDICES:

Soto AM, Maffini MV, Calabro JM, Wieloch C, Sonnenschein C. Mammary gland stroma is responsible for epithelial cell neoplasia. Differentiation 70:321, 2002

Maricel V. Maffini, Ana M. Soto, Janine M. Calabro, Angelo A. Ucci and Carlos Sonnenschein, The stroma as a crucial target of chemical carcinogens in the rat mammary gland. Journal of Cell Science, 117, 1495-1502, 2004.

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Maffini MV, Calabro JM, Soto AM, Sonnenschein C. Stromal regulation of neoplastic development: Age-dependent normalization of neoplastic mammary cells by mammary stroma. American Journal of Pathology. In Press 2005

MAMMARY GLAND STROMA IS RESPONSIBLE FOR EPITHELIAL CELL NEOPLASIA

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Mammary gland development is driven by a network of signals between stroma and epithelium. The tissue organization field theory of carcinogenesis (TOFT) proposes that altered reciprocal interactions between stroma and epithelium initiate the neoplastic process. We assessed whether the primary target of the carcinogen N-nitroso-methylurea (NMU) in mammary glands of Wistar-Furth rats is the epithelium, the stroma or both. The 4th and 5th mammary gland fat pads were cleared of epithelium (CFP) at 21 days of age. One month later, these animals were treated with NMU (Groups 1 and 2) or vehicle (3 and 4). One week later, vehicle-treated epithelial cells were transplanted into the CFP of Groups 1 and 4 while NMU-treated epithelial cells were transplanted into the CFP of Groups 2 and 3. Also, positive and negative controls consisting of intact virgin rats injected respectively with NMU (Group 5), and vehicle (Group 6) were included. Tumors appeared in Group 1 (92.8%), 2 (75%) and 5 (100%) and were absent in Groups 3, 4 and 6. Whole mount preparations and histology confirmed the mammary tumor origin of the palpable lesions. Our results suggest that only the stroma is the target of the carcinogen. This novel concept in carcinogenesis should provide for a more rational study of breast cancer.

The stroma as a crucial target in rat mammary gland carcinogenesis

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Summary

A complex network of interactions between the stroma, the extracellular matrix and the epithelium drives mammary gland development and function. Two main assumptions in chemical carcinogenesis of the mammary gland have been that carcinogens induce neoplasia by causing mutations in the DNA of the epithelial cells and that the alterations of tissue architecture observed in neoplasms are a consequence of this primary mutational event. Here, we use a rat mammary tissue recombination model and the chemical carcinogen N-nitrosomethylurea (NMU) to determine whether the primary target of the carcinogen is the epithelium, the stroma or both tissue compartments. Mammary epithelial cells were exposed in vitro either to the carcinogen or vehicle before being transplanted into the cleared fat pads of rats exposed to carcinogen or vehicle. We observed that neoplastic transformation of these mammary epithelial cells occurred only when the stroma was exposed in vivo to NMU, regardless of whether or not the epithelial cells were exposed to the carcinogen. Mammary epithelial cells exposed in vitro to the carcinogen formed phenotypically normal ducts when injected into a non-treated stroma. Mutation in the Ha-ras-1 gene did not correlate with initiation of neoplasia. Not only was it often found in both cleared mammary fat pads of vehicle-treated animals and intact mammary glands of untreated animals, but it was also absent in some tumors. Our results suggest that the stroma is a crucial target of the carcinogen and that mutation in the Ha-ras-1 gene is neither necessary nor sufficient for tumor initiation.

Key words: Mammary carcinogenesis, Stroma, Neoplasms, N-nitrosomethylurea, NMU, Ha-ras-1 mutation, Tissue architecture

Introduction

A comprehensive understanding of carcinogenesis in general, and in the rat mammary gland in particular, has been delayed because of epistemological issues. It has been obvious to many of us working in the field of carcinogenesis that we lack a consistently reliable set of premises on which we can base a solid rationale to conduct research (Sonnenschein and Soto, 1999a; Sonnenschein and Soto, 2000; Moss, 2003). For almost a century, a majority of researchers have followed the lead provided by Theodor Boveri in 1914, favoring the notion that carcinogenesis occurs at the cellular level of biological organization (Boveri, 1929). After a number of course corrections to accommodate lacks of fit, Boveri's ideas have coalesced into what is now generally accepted as the Somatic Mutation Theory of carcinogenesis (Hanahan and Weinberg, 2000; Mastorides and Maronpot, 2002). Throughout the twentieth century, this theory has been challenged by others, who proposed instead that carcinogenesis takes place at the tissue level of biological organization (Orr, 1958; Smithers, 1962; Hodges et al., 1977; Sonnenschein and Soto, 2000). In the past decade, attempts to find a synthetic position that would incorporate claims from both theoretical approaches have also been advanced (Folkman et al., 2000; Bissell and Radisky, 2001; Thiery, 2002). Objectively, however, the identification of the target(s) upon which the carcinogenic agents act in order to initiate neoplastic transformation has, so far, remained elusive.

The development of mammary cancer in susceptible rodent strains following administration of N-nitrosomethylurea (NMU) is a widely accepted model for the study of chemical carcinogenesis (Gullino et al., 1975). The majority of NMU-induced rat mammary tumors are carcinomas or adenocarcinomas, that is tumors of presumed epithelial origin (Thompson, H. J. et al., 2000a). According to the Somatic Mutation Theory, a neoplastic outcome would result from accumulated NMU-induced mutations in one of the epithelial cells of this gland (Guzman et al., 1992; Gould, 1995). Although these carcinomas show an altered organization of both the epithelium and the stroma, when examined through a light microscope, changes observed in the stroma have been assumed to be a secondary effect of the primary mutational events in the epithelium.

An alternative theory considers that carcinogenesis is a process akin to development gone awry (Pierce et al., 1978; Sonnenschein and Soto, 1999a). The Tissue Organization Field Theory proposes that carcinogens alter stromal-epithelial interactions and that proliferation is the default state of all cells (Sonnenschein and Soto, 1999b; Sonnenschein and Soto, 2000). Carcinogenesis would therefore be an emergent phenomenon that takes place at the tissue level of biological organization. As mentioned above, several authors have proposed synthetic approaches that straddle both theories as applied to mammary carcinogenesis (Bissell and Radisky, 2001; Wiseman and Werb, 2002; Thiery, 2002).

In an effort to deal comprehensively and simultaneously with all the competing theories, we decided to use a rat mammary tissue recombination model. This model affords an easy surgical separation of stroma and epithelium such that each compartment might be exclusively exposed to the carcinogen. We chose NMU because it is a direct carcinogen in that it does not need to be metabolized in order to form DNA adducts and has a very short half-life (Swann, 1968). This minimizes the risk of inadvertent indirect exposure of epithelial cells to the carcinogen when recombining them with the stroma. The outcome of the proposed experimental design would determine whether the primary target of NMU is the epithelium (as suggested by the Somatic Mutation Theory), the stroma (as implied by the Tissue Organization Field Theory), or both tissue compartments.

Materials and Methods

Chemicals and cell culture reagents

NMU (CAS #684-93-5), insulin, penicillin, progesterone, prolactin, fatty acid-free fraction V bovine serum albumin (BSA), hydrocortisone, human transferrin, ascorbic acid, gentamicin, aluminum potassium sulfate and methyl salicylate were purchased from Sigma-Aldrich. Human epidermal growth factor (EGF) and Matrigel™ were obtained from Becton Dickinson. Phenol red-free DMEM/F12 medium and trypsin were obtained from Gibco. Collagenase was purchased from Worthington Biochemical Corporation and pronase from Calbiochem. Percoll™ was obtained from Amersham Pharmacia Biotech and Carmine from Fisher Scientific.

Animals

Wistar-Furth rats were purchased from Harlan and housed with food and water ad libitum. Animals were maintained on a 14:10 hours light:dark cycle and care was in accordance with the Guidelines for the Care and Use of Animals and the Tufts-New England Medical Center Institutional Animal Care and Use Committee. When the animals were 21 days old, the mammary epithelium was surgically removed from the 4th and 5th inguinal mammary glands according to procedures outlined previously (DeOme et al., 1959). In each of the animals used in these experiments, the excised epithelium was whole-mounted and observed microscopically to assure that the ductal tree was removed in its entirety and that only a small portion of the fat pad remained attached to it (Fig. 1A).

Tissue recombination experimental design

The animals with cleared fat pads were distributed into experimental Groups 1-4. At 52 days of age, animals from Groups 1 and 2 received a single intraperitoneal dose of 50 mg NMU/kg body weight dissolved

Fig. 1. (A) Whole-mount preparation of an intact mammary gland from a 21-day-old rat showing the ductal tree and lymph nodes. (B) Mammary gland fat pad cleared of epithelium at 21 days of age and excised at the end of the experiment, 11 months later. Bar, 4 mm.

in warm 0.85 g/l NaCl solution (vehicle), pH 5.0; by contrast, Groups 3 and 4 were exposed to just the vehicle. Five days later, 50,000 mammary epithelial cells were injected into the cleared fat pads according to the following experimental design: animals from Groups 1 and 4 received vehicle-treated mammary epithelial cells, and animals from Groups 2 and 3 received NMU-treated mammary epithelial cells. Positive and negative control groups were used. These control groups were intact virgin animals that were age-matched to the animals in Groups 1 to 4. They were not subjected to any surgical manipulation. These animals were treated at 52 days of age with NMU and vehicle, respectively. They were injected at the same time with the animals used in Groups 1 to 4. Intact animals injected with NMU were considered as the positive control for tumor incidence and histopathology of the tumors (Group 5). Animals injected with vehicle were considered as the control for spontaneous tumors and for the normal architecture of the mammary gland (Group 6). Four experiments were performed where all the experimental groups were represented. Animals were excluded from the analyses when no epithelial outgrowths were found in the whole mounts ('no takes') or if they died as a result of surgical complications. The initial (i) and final (f) sample sizes at 9 months after the NMU injection were as follows: Group 1, i=14, f=13; Group 2, i=10, f=8; Group 3, i=12, f=10; Group 4, i=11, f=6.

Cleared fat pad repopulation

A second set of animals with cleared fat pads was transplanted with 50,000 mammary epithelial cells at 52 days of age. The recombinants were inspected 30, 60 and 90 days later.

Mammary epithelial cell culture

Mammary epithelial cells were isolated from 50-60-day-old virgin female Wistar-Furth rats using a combination of two previously described protocols (Hahm and Ip, 1990; Imagawa et al., 2000). Briefly, the 4th and 5th inguinal mammary glands were bilaterally excised, minced and digested in phenol red-free DMEM containing 0.15% collagenase III at 37°C for 2 hours with agitation. This digest was centrifuged and the pellet was then treated with 0.05% pronase for 30 minutes at 37°C with agitation. This suspension was filtered through a 530 μm-pore Nitex® filter (Sefar America) and the filtrate was centrifuged at 100 g for 3 minutes (Hahm and Ip, 1990). The pellet was resuspended in 1-2 ml of serum-free medium (SFM) containing phenol red-free DMEM/F12 plus 10 μg/ml insulin, 1 μg/ml progesterone, 10 ng/ml EGF, 1 μg/ml prolactin, 1 mg/ml BSA, 1 μg/ml hydrocortisone, 5 μg/ml human transferrin, 0.88 μg/ml ascorbic acid and 50 μg/ml gentamicin (Hahm and Ip, 1990). This cell suspension was layered over a pre-made Percoll™ gradient (Imagawa et al., 2000) and centrifuged for 20 minutes at 800 g. Single epithelial cells and organoids were recovered from the gradient, diluted in SFM and similarly centrifuged. The pellet was resuspended in SFM and plated on Matrigel™-coated (100 μg/cm²) 6-well plates (Becton Dickinson). This layer was enough to promote cell attachment but insufficient to facilitate three-dimensional growth. Non-epithelial cells were successfully removed by treating the plates with a 0.025% trypsin and 0.01% EDTA solution. Five days before being transplanted into recipient animals, the mammary epithelial cells were treated with SFM containing either vehicle or 50 μg/ml NMU for 1 hour at 37°C (Miyamoto et al., 1988). The cells were then rinsed twice with SFM and fresh SFM was added. NMU was used within 5 minutes of preparation. A different batch of mammary epithelial cells prepared following this protocol was used for each of the four experiments. The dose of NMU used in the in vitro experiments was selected following Miyamoto et al. (Miyamoto et al., 1988).

Epithelial cell transplantation

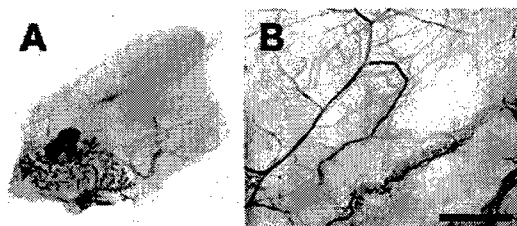After harvesting by trypsinization, the cells were counted in a Coulter

Counter Apparatus (Model ZM, Coulter Electronics). The volume of the cell suspension was adjusted in order to inject 50,000 cells in 10 μl into the CFP using a Hamilton syringe. All rats receiving a cell transplant were palpated weekly, starting one month after the mammary epithelial cell inoculation. Thoracic glands were used as internal controls for the carcinogen and were equally palpated. Animals were sacrificed when inguinal tumors reached 1 cm in diameter or 9 months after cell transplant, whichever came first.

DNA extraction and analysis of Ha-*ras*-1 gene mutation

DNA was extracted from mammary neoplastic lesions, fat pads and whole mammary glands from virgin rats using the DNeasy kit (Quiagen), following the manufacturer's instructions. We used the mismatch amplification mutation assay (MAMA) described by Cha et al. (Cha et al., 1996) with some modifications. The MAMA is specific for the codon 12 GGA to GAA mutation in Ha-*ras*-1 gene. Briefly, this method uses two sets of primers; one targets the mutation and the other a control area in the genomic DNA. The mutant-specific mismatch primer PAA (5'-CTTGTGGTGGTGGGCGCTGAA-3'), the Pmnl2 (5'-ACTCGTCCACAAAATGGTTC-3') and the control primers (P1: 5'-CCTGGTTTGGCAACCCCTGT-3'; and Pmnl2: 5'-ACTCGTCCACAAAATGGTTC-3') were used at a 40 ng/μl concentration. The PCR was performed using Platinum Supermix (Invitrogen). The PCR products were run in a 2% agarose gel (Gibco). The expected size of the non-mutated Ha-*ras*-1 gene is 128 bp, whereas the mutated Ha-*ras*-1 gene is 74 bp.

Whole mounts and histology

Whole mounts were prepared following protocols described by the Laboratory of Genetics and Physiology at the National Institute of Diabetes, Digestive and Kidney Diseases within the National Institutes of Health (http://mammary.nih.gov), and Thompson et al. (Thompson et al., 1995). The mammary glands were removed and spread on a 75×50×1 mm glass slide (Fisher Scientific), fixed overnight in 10% phosphate-buffered formalin, dehydrated in 70%, 95% and 100% alcohols, cleared in toluene, rehydrated and stained with Carmine Alum. After staining, the whole mounts were dehydrated as described above, cleared in xylene, and bagged in Kpak® SealPak heat-seal pouches in methyl salicylate. The whole mounts were analyzed under a stereomicroscope for microscopic lesions. Tumors larger than 0.5 cm were removed before whole mounts were prepared and separately fixed as described above. Microscopic lesions were removed and embedded in paraffin for histological analysis. Images were captured with an AxioCam HR color digital camera (Carl Zeiss) attached to a Stemi 2000 stereomicroscope (Carl Zeiss).

Immunohistochemistry

An antigen-retrieval method based on microwave pretreatment and 0.01 M sodium citrate buffer (pH 6) was used as previously described (Maffini et al., 2001). Mouse monoclonal anti-pan cytokeratin (Sigma-Aldrich), anti-vimentin (Novocastra) and anti-desmin (Novocastra) were used at 1:700, 1:100 and 1:100 dilutions, respectively. The antigen-antibody reaction was visualized using the streptavidin-peroxidase complex, with diaminobenzidine tetrahydrochloride (Sigma-Aldrich) as the chromogen. Counterstaining was performed with Harris' hematoxylin. For the double-staining immunofluorescence technique, cytokeratin and vimentin were detected using a previously described technique (Maffini et al., 2002). The primary antibodies were used at 1:100 dilutions in 4% BSA supplemented with 10% normal goat serum. Secondary antibodies and streptavidin-Alexa 594 and 488 (Molecular Probes) were used at 1:100 dilutions. Cell nuclei were counterstained with Hoechst 33258. Images were captured with an AxioCam HR

color digital camera (Carl Zeiss) attached to an Axioskop 2 plus microscope (Carl Zeiss).

Statistics

Statistical significance of the incidence of neoplastic lesions and Ha-*ras*-1 gene mutation were determined using the χ^2 Test. The Mann-Whitney and Kruskal-Wallis tests were used to compare the latency periods and the number of lesions in inguinal and thoracic mammary glands between groups. To compare the latency of pectoral and inguinal lesions in the same animal within each treatment group, we used the Wilcoxon signed ranks test, and treated the pectoral and inguinal latency for each animal as a pair.

Results

Normal ducts developed from cultured mammary epithelial cells

The tissue recombination components were mammary gland stroma (cleared fat pad) and mammary epithelial cells grown in vitro (Fig. 2A). We observed the phenotype of the ductal outgrowth and the repopulation dynamics in the cleared fat pads after transplantation of 50,000 mammary epithelial cells. The ductal outgrowths were phenotypically normal and, 90 days after mammary epithelial cell transplantation, the ductal tree covered a third of the fat pad (Fig. 2B-E).
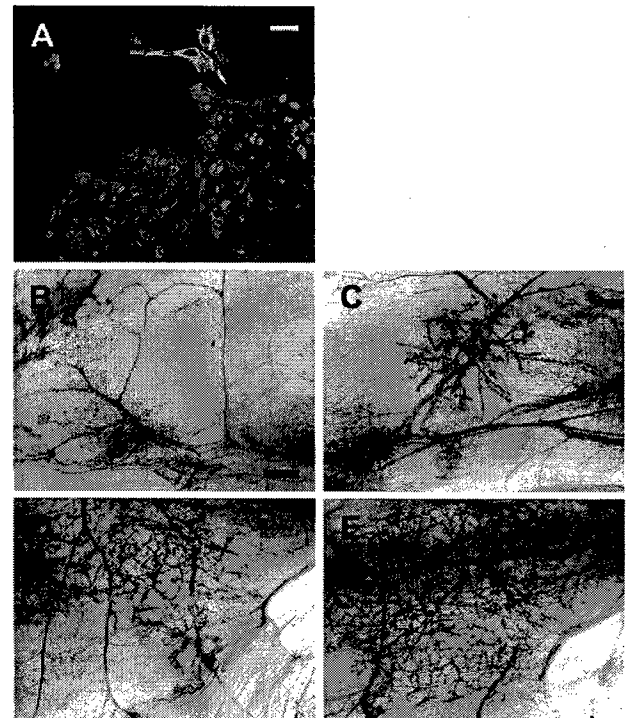
Fig. 2. Repopulation of the mammary gland. (A) Mammary epithelial cells grown in culture showing the expression of cytokeratin (red) and vimentin (green). Mammary epithelial cells averaged 90% of the total cell population transplanted into cleared fat pads. Counterstaining, Hoescht 33258 (blue). Mammary epithelial cells were injected into cleared fat pads and the recombinants were harvested at 0 (B), 30 (C), 60 (D) and 90 (E) days after cell injection. Bars, 20 μm (A); 2 mm (B-E).

Neoplastic transformation of mammary epithelial cells

We observed that only those animals whose stroma was exposed to NMU developed neoplasms, regardless of whether or not the transplanted mammary epithelial cells were exposed to the carcinogen (Fig. 3A). The incidence of neoplastic lesions in Groups 1 and 2 was 76.9% (10/13) and 75% (6/8), respectively (Fig. 3B). The positive control Group 5 had 100% incidence (6/6). There were no significant differences in neoplastic incidence between Groups 1 and 2 ($P=0.920$) or between Groups 1 or 2 and Group 5 ($P=0.200$ and $P=0.186$, respectively). By contrast, the animals whose stroma was
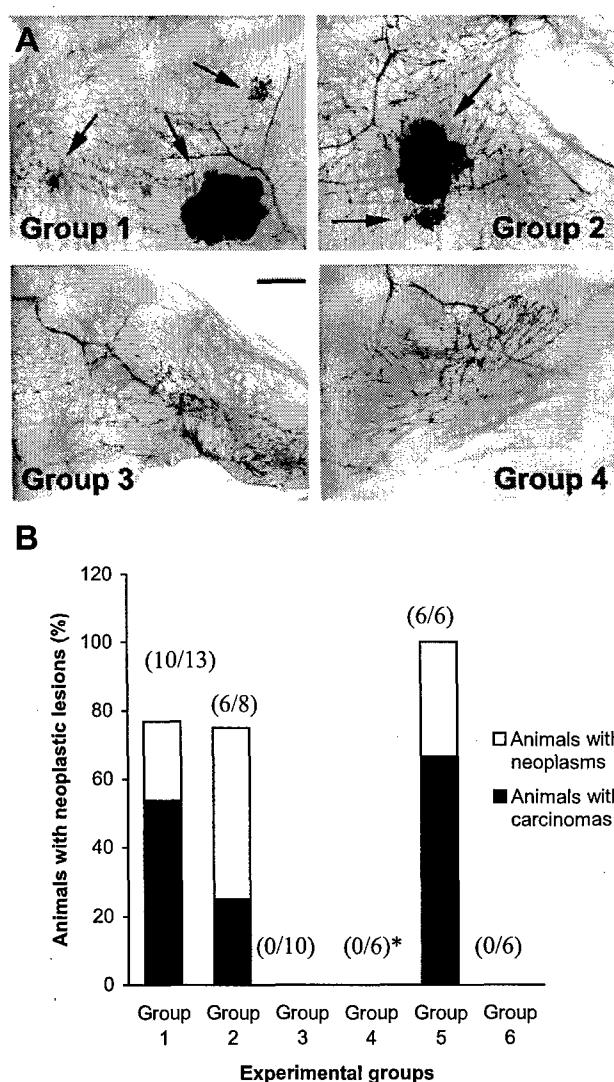
exposed to vehicle developed no neoplasms, regardless of whether the mammary epithelial cells were exposed in vitro to NMU (Group 3, 0/10) or to vehicle (Group 4, 0/6). The negative control Group 6 had 0% incidence (0/6). Group 3 was significantly different from Group 1 ($P<0.001$) and Group 2 ($P=0.001$). Group 4 was also significantly different from Group 1 ($P=0.002$) and Group 2 ($P=0.005$).

Multiple neoplastic lesions were found

Multiple lesions were observed in the inguinal mammary glands of rats in Groups 1, 2 and 5 (Fig. 4A), suggesting that the neoplasms found in these groups were not a consequence of mechanical injury resulting from the injection procedure. The inguinal glands of Group 5 had twice as many lesions as those in Groups 1 and 2 ($P=0.013$ and $P=0.001$, respectively) (Fig. 4A). This difference might have been owing to the fact that the intact mammary glands in Group 5 had a full complement of epithelium whereas Groups 1 and 2 had an

Fig. 3. Neoplasms developed in NMU-treated stroma only. (A) Mammary gland whole-mount preparations show abnormal outgrowths in animals whose cleared fat pads were exposed to NMU prior to recombination with vehicle-treated mammary epithelial cells (Group 1) or NMU-treated mammary epithelial cells (Group 2). Neoplastic lesions (arrows) were confirmed histologically. Groups 3 and 4 developed normal-like ductal outgrowths. Bar, 2 mm. (B) Percentage of neoplastic lesions and incidence of carcinomas per experimental group. The number of rats with neoplastic lesions out of the total number of animals in each group is indicated in parenthesis. *See Materials and Methods for further details.
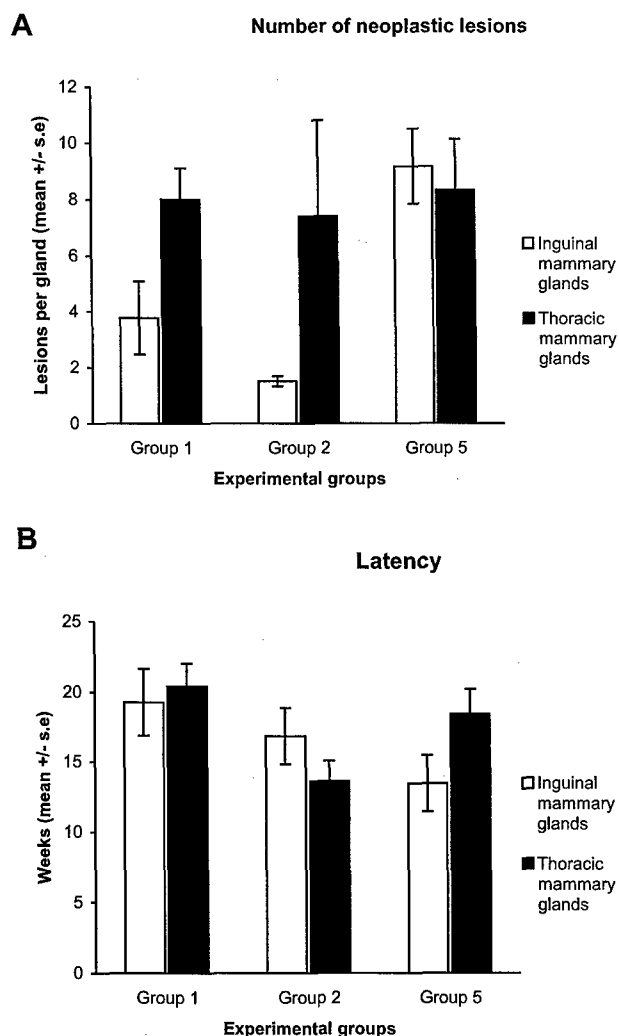

Fig. 4. Incidence of neoplasms and latency period. (A) Number of neoplastic lesions in inguinal and thoracic mammary glands (mean±s.e.). (B) Latency of neoplastic lesions in inguinal and thoracic mammary glands expressed in weeks (mean±s.e.).

initial population of only 50,000 mammary epithelial cells. The incidence of neoplastic lesions in the thoracic mammary glands of NMU-treated rats from Groups 1, 2 and 5 was comparable (P=0.622) (Fig. 4A). There was no significant difference among Groups 1, 2 and 5 regarding inguinal tumor latency periods (P=0.147). The latency period was similar in the thoracic and inguinal mammary glands within the same experimental groups (Group 1: P=0.276; Group 2: P=0.414; Group 5: P=0.684) (Fig. 4B).

We performed the histopathological analyses of the neoplastic lesions following the classification described by Russo et al. (Russo et al., 1990). Carcinomas were seen in 53.8% of the animals from Group 1, 25% of Group 2 and 66.7% of Group 5 (Fig. 3B), and represented 70%, 33% and 66.7% of the neoplasms found in these groups, respectively (Table 1). The most frequent type of neoplastic lesion was papillary carcinoma (Fig. 5B, Table 1). All the tumors were of epithelial origin; the neoplastic cells were cytokeratin positive, and vimentin and desmin negative (Fig. 5C). Regardless of whether or not the mammary epithelial cells had been exposed to NMU, the tissue-recombined mammary glands of animals that did not develop tumors appeared histologically similar to a normal mammary gland (Fig. 5A).

Mutated Ha-*ras*-1 does not correlate with neoplasia

We analyzed the DNA of neoplastic lesions from Groups 1 and 2 and observed that 2 out of 11 neoplasms from Group 1, and 1 out of 6 from Group 2, lacked the G-A mutation in the codon 12 of the Ha-*ras*-1 gene. Similarly, DNA taken from the neoplastic lesions in the positive control (Group 5) showed that 1 out of 7 lacked the point mutation (Fig. 6). No statistically significant difference was found between the groups (P=0.977). In order to test whether any correlation existed between the presence of the mutated Ha-*ras*-1 gene and the initiation of neoplasia, we analyzed DNA extracted from the stroma of animals treated with vehicle (i.e. Groups 3 and 4). All stroma samples from Groups 3 (7 out of 7) and 4 (6 out of 6) showed the mutation. Thus, we now report that this Ha-*ras*-1 gene mutation was present in the mammary gland fat pad of rats exposed to vehicle. Moreover, DNA harvested from whole mammary glands of intact rats randomly taken from our colony

Fig. 5. Sections of recombinant tissues. (A) Section from a recombinant of vehicle-exposed stroma and NMU-exposed mammary epithelial cells. The histoarchitecture resembles a normal mammary gland. (B) Papillary carcinoma from a recombinant of NMU-exposed stroma and vehicle-exposed mammary epithelial cells. Hematoxylin and eosin staining (A,B). (C) Immunohistochemistry for cytokeratin, vimentin and desmin in sections of the tumor shown in B. Counterstaining: Harris' hematoxylin. Bar, 100 μm.

Table 1. Incidence of mammary neoplastic lesions in groups exposed to NMU

Experimental group	Histopathological classification	Incidence (%)*
Group 1	Carcinomas	70
	Papillomas	10
	Cystoadenomas	10
	Adenomas	10
Group 2	Carcinomas	33.3
	Papillomas	16.7
	Fibroadenomas	33.3
	Fibroma	16.7
Group 5 (Positive control)	Carcinomas	66.7
	Adenomas	16.7
	Cystoadenomas	16.6

*The number of neoplastic lesions in each histological category was divided by the total number of lesions observed in each experimental group
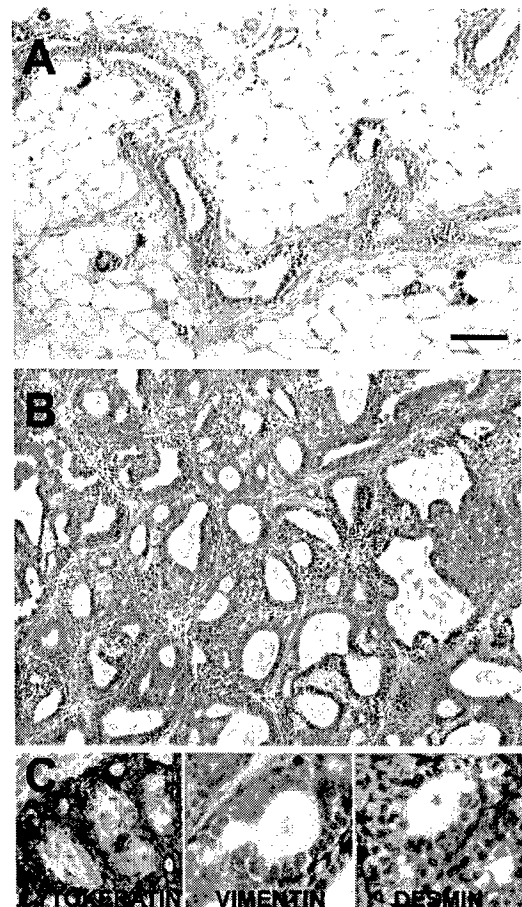
(4 out of 4) also showed the mutation, which agrees with previous findings (Cha et al., 1996). The incidence of mutated Ha-*ras*-1 gene was not significantly different between animals that were or were not exposed to NMU (P=0.604). Finally, the mutation was also assessed in DNA isolated from mammary epithelial cells, mammary fibroblasts, and mammary pre-adipocytes collected from intact virgin rats and grown in vitro. All these different types of cells were collected at different times during the course of 2 years. DNA was extracted from frozen cells, vehicle-treated cells and NMU-treated cells. The presence of the mutation did not correlate with cell type, culture conditions or carcinogen treatment (data not shown).

Discussion

Our results regarding the role of histoarchitecture in carcinogenesis are consistent with previous findings stemming from the use of diverse rodent models. Barcellos-Hoff and
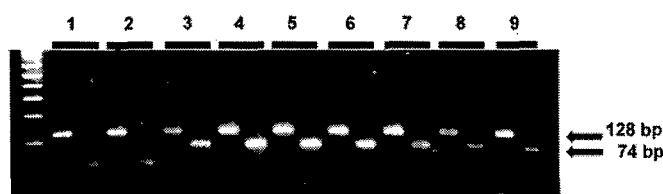
Fig. 6. Analysis of the presence of point mutation in the Ha-*ras*-1 gene using the MAMA. The mutant-specific amplification product is 74 bp whereas the normal product is 128 bp. Lanes 1, 5, 6 and 7: mammary tumors from Group 5. Tumor in lane 1 lacks Ha-*ras*-1 gene mutation. Lane 2: mammary tumor from Group 1. Note absence of Ha-*ras*-1 gene mutation. Lanes 3 and 4: mammary tumors from Group 2. Lanes 8 and 9: normal mammary tissue from intact animals taken randomly from the colony. Note: the smaller bands in lanes 1 and 2 correspond to dimers of the primer.

Ravani showed that radiation-induced changes in the stromal microenvironment contributed to the neoplastic progression of non-irradiated, quasi-normal, established COMMA-1 mammary epithelial cells (Barcellos-Hoff and Ravani, 2000). Sternlicht et al. observed that overexpression of the matrix metalloproteinase stromelysin-1 can induce carcinogenesis in mouse mammary glands (Sternlicht et al., 1999). Also, using tissue recombinant techniques, Olumi et al. concluded that 'primary, phenotypically normal fibroblasts associated with a human epithelial malignancy can stimulate progression of a nontumorigenic (prostate) epithelial cell' (Olumi et al., 1999). Thompson et al. have also used a tissue recombination model, the mouse prostate reconstitution model system, and observed that 'intrinsic properties of the BALB/c mesenchyme can arrest the progression of ras+myc-initiated C57BL/6 epithelium from benign hyperplasia to malignant carcinoma' (Thompson et al., 1993).

Our experiments, designed to explore simultaneously the competing theories mentioned in the introduction, suggest that the stroma is a target of NMU in mammary carcinogenesis. We were concerned, of course, that inadvertent technical mishaps might have influenced our data. For instance, epithelial cells might have remained in the fat pads after the clearing procedure and could have been exposed in vivo to NMU. We addressed this possibility by microscopically examining the tissue containing the ductal tree after clearing the fat pads at 21 days of age and verifying that the margins contained no epithelial cells (Fig. 1A). In addition, we also cleared the 5th mammary gland to prevent the migration of indigenous epithelial cells into the 4th cleared fat pad. Therefore, we consider it unlikely that epithelial cells were present after clearing. It was also reassuring to observe that cleared fat pads not injected with mammary epithelial cells remained free of epithelium at the end of the experiment (Fig. 1B).

Several research groups have used experimental rodent models to explore the concept that epithelial cells are the targets of carcinogens, as implied by the Somatic Mutation Theory. Miyamoto et al. reported tumor formation after mammary epithelial cells were exposed to NMU in vitro and injected into cleared fat pads (Miyamoto et al., 1988). These authors used a cell inoculum one order of magnitude higher than the one we used and a different cell purification method. Also, they added the NMU when the cultures were 3 days old

and made no reference to the degree of fibroblast contamination. It is conceivable that, in their experiments, fibroblasts exposed to NMU in vitro could have played a role in the carcinogenic process. On the contrary, we repeatedly trypsinized and subcultured the mammary epithelial cells to enrich this pool of cells and reduce fibroblast contamination. In essence, we exposed a highly enriched mammary epithelial cell population to NMU (Fig. 2A). Furthermore, Miyamoto et al. injected the mammary epithelial cells into fat pads immediately after clearing, while in the midst of wound healing (Miyamoto et al., 1988). In this context, it has been shown that carcinogenesis is promoted by a wounded stroma (Konstantinidis et al., 1982; Sieweke et al., 1990). Their data and those by Guzman et al. do not suggest a positive correlation between tumor yield and either NMU concentration or the number of exposures to this carcinogen in vitro. Moreover, normal epithelial outgrowths were observed at all NMU doses (Guzman et al., 1987; Miyamoto et al., 1988). Using yet another protocol, Kamiya et al. showed that NMU- or radiation-exposed mammary epithelial cells yielded mammary carcinomas when grafted into rat fat pads that were 'cleared' by injecting 70% ethanol (Kamiya et al., 1995). They interpreted these data as evidence that tumor formation was due to undefined epigenetic factors rather than to mutations. They also observed that tumor incidence diminished as the number of cells injected increased, an outcome inconsistent with the Somatic Mutation Theory.

These experiments dealing with in vitro exposure to NMU were based on the premise that NMU acted directly on the epithelial cells and, therefore, under this rationale, no attempt was made to evaluate the role of the stroma in tumor formation (Greiner et al., 1983; Guzman et al., 1987; Miyamoto et al., 1988; Delp et al., 1990). The novelty of our observations stems from the fact that a carcinogen-treated stroma was able to transform vehicle-treated cells into neoplastic tissues comparable with those seen in intact NMU-exposed rats (positive control Group 5) (Fig. 5 and Table 1).

The prevalent hypothesis that NMU exposure results in carcinogenesis because of NMU-induced point mutations in the codon 12 of the Ha-*ras*-1 gene of mammary epithelial cells (Zarbl et al., 1985) has been challenged. As shown in our results and in the literature (Cha et al., 1994; Cha et al., 1996; Swanson et al., 1996; Shirai et al., 1997; Thompson, T. A. et al., 2000b), not all NMU-induced mammary neoplasms express this mutation. Also, Korkola and Archer have observed comparable results in NMU-induced pre-neoplastic lesions (Korkola and Archer, 1999). Equally important, this mutation is present in mammary glands from non-exposed animals (Cha et al., 1996). Here, we confirm these findings and show that the frequency of tumors expressing mutated Ha-*ras*-1 is statistically similar in the positive controls (Group 5) and recombinants from NMU-exposed stroma (Groups 1 and 2). Moreover, we also observed that mutated Ha-*ras*-1 is also present in the cleared mammary fat pad of vehicle-exposed animals. Furthermore, Zhang et al. demonstrated that increasing the dose of NMU increased total tumor yield but reduced the frequency of mammary tumors expressing mutated Ha-*ras*-1 (Zhang et al., 1990). In sum, these data suggest that the Ha-*ras*-1 gene mutation appears to be neither necessary nor sufficient for neoplastic transformation and that it is not exclusively present in the epithelial cells.

The concept that altered tissue architecture is at the core of carcinogenesis was pioneered by Waddington (Waddington, 1935), Orr (Orr, 1958) and, more recently, by Bissell and Radisky (Bissell and Radisky, 2001) and others (Sonnenschein and Soto, 2000; Moss, 2003, Weaver and Gilbert, 2004). Altogether, our data and those of others challenge the long-held notion that carcinogens induce mammary cancer by causing mutations in the DNA of an epithelial cell (Fearon and Vogelstein, 1990; Mastorides and Maronpot, 2002). These results suggest the need to explore the roles that the stroma components [i.e. the cells (fibroblasts, adipocytes, mast cells, etc.)] and the extracellular matrix play in rodent mammary carcinogenesis. Efforts should also be directed at exploring the role of the stroma in experimental models for carcinogenesis involving organs other than the mammary gland (i.e. skin, prostate, liver, bladder). To accommodate a novel perspective on the role of the stroma in carcinogenesis, a rigorous analysis of concepts, definitions and experimental approaches is now needed. This will facilitate the identification of the mediators responsible for the altered tissue phenotype in cancers and of ways to reverse their effect by adopting a solid epigenetic perspective.

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The somatic mutation theory of cancer: growing problems with the paradigm?

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Summary

The somatic mutation theory has been the prevailing paradigm in cancer research for the last 50 years. Its premises are: (1) cancer is derived from a single somatic cell that has accumulated multiple DNA mutations, (2) the default state of cell proliferation in metazoa is quiescence, and (3) cancer is a disease of cell proliferation caused by mutations in genes that control proliferation and the cell cycle. From this compelling simplicity, an increasingly complicated picture has emerged as more than 100 oncogenes and 30 tumor suppressor genes have been identified. To accommodate this complexity, additional ad hoc explanations have been postulated. After a critical review of the data gathered from this perspective, an alternative research program has been proposed. It is based on the tissue organization field theory, the premises of which are that carcinogenesis represents a problem of tissue organization, comparable to organogenesis, and that proliferation is the default state of all cells. The merits of these competing theories are evaluated herein. *BioEssays* 26:1097–1107, 2004. © 2004 Wiley Periodicals, Inc.

Introduction

The somatic mutation theory of carcinogenesis (SMT) deals with sporadic cancers, which represent over 95% of all cancers. The SMT has been the prevailing paradigm in cancer research for the last 50 years.[1–3] Its main premise claims that cancer is derived from a single somatic cell that has accumulated multiple DNA mutations over time. This implies that cancers are monoclonal, i.e., they are all derived from a single faulty, mutated cell.[4] A second implicit premise is that in the absence of regulatory stimuli, metazoan cells in situ are proliferatively quiescent.[5] In other words, the default state of cell proliferation in multicellular organisms is quiescence (See Ref. 6, pp 1–13). A third premise of this theory considers that cancer is a disease of cell proliferation and that

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cancer-causing mutations occur in genes that control cell proliferation and/or the cell cycle.[7,8]

On what bases have research programs favored the SMT as the main theory of carcinogenesis? First, a sizable number of carcinogenic chemicals were found to be mutagenic. Second, specific genes in so-called tumor viruses (called "transforming" genes) enabled such phenomena as in vitro transformation and the development of tumors at the injection site in some animal models. Next was the discovery that these transforming genes, or oncogenes, were homologous to genes present in non-infected cells. This shifted the search for exogenous genetic causes to endogenous ones and brought the role of DNA mutations back to prominence, now as cellular oncogenes, or proto-oncogenes. The major event in this unifying process was probably data showing that DNA fragments from chemically transformed cells were in turn able to transform recipient cells.[9] Finally, the DNA sequences involved were identified as mutated versions of the endogenous, "normal" cellular genes. A series of observations relating these oncogenes to growth factor receptors and to signal transduction pathways bolstered this updated version of the SMT. The implications of these findings were 1) that the products of the mutated oncogenes were activated and 2) that their activation led to increased cell proliferation.

Thus, oncogenes were considered gain-of-function mutations that led the cells harboring these mutants to enhanced proliferation. This latter concept strengthened the research program on signal transduction and, consequently, resulted in a staggering contribution of knowledge in the biochemistry of cellular processes.

Meanwhile, the study of familial, hereditary cancers (about 5% of all human cancers) revealed that the DNA defects transmitted along the germline were due to deletions in specific genes. Unlike the oncogenes, these deletions implied a loss-of-function. The first of these anti-oncogenes (later dubbed "tumor suppressor" genes) was the retinoblastoma gene (Rb), which was soon implicated in the regulation of cell division. Thus, mutations affecting cell cycle regulatory genes became a major cancer research topic.

The initial appeal of the oncogene theory was its simplicity, an assumption later challenged by the increasingly complicated picture that emerged after two decades of intensive research. To date, more than 100 oncogenes and more than

30 tumor suppressor genes have been identified. As summarized in a recent review by Hahn and Weinberg: "For those who believe in the simplification and rationalization of the cancer process, the actual course of research on the molecular basis of cancer has been largely disappointing. Rather than revealing a small number of genetic and biochemical determinants operating within cancer cells, molecular analyses of human cancers have revealed a bewilderingly complex array of such factors."[10] To overcome these shortcomings they proposed searching for unifying rules governing the behavior of cancer cells, such as, "...[the abilities] to generate their own mitogenic signals, to resist exogenous growth-inhibitory signals, to evade apoptosis, to proliferate without limits (i.e., to undergo immortalization), to acquire vasculature (i.e., to undergo angiogenesis), and in more advanced cancers, to invade and metastasize."[10] Thus, additional research has been proposed albeit within the same paradigm that cancer is a phenomenon explicable at the cellular level of biological organization.

In this article, we will critically review the data collected under the somatic mutation paradigm to explain sporadic cancers and then offer an alternative research program centered on the premises that carcinogenesis represents a problem of tissue organization and that proliferation is the default state of all cells.

What is a neoplasm?

Literally, neoplasm means new growth. Pathologists have tried to define neoplasms for a century. Their definitions were unsatisfactory since properties attributed to "cancer cells" were also present in normal cells. Because tumor size increases with time, researchers have considered that the underlying cause must have been either excessive or autonomous cell proliferation.[11] However, in addition to the accumulation of cells, the hallmark of neoplasms is altered tissue organization.[12] Pathologists examine tissue samples using light microscopes to unambiguously diagnose neoplasms. For the most part, neoplasms retain the distinctive structures that characterize the organ of origin. Like normal organs, neoplasms also contain a parenchyma (the distinctive cell type of an organ) and a supporting tissue or stroma. For their normal development and function, tissues require a normal architecture where parenchymal and stromal constituents operate in a coordinated way through reciprocal interactions. A principal aim of cancer research is to elucidate the mechanisms by which neoplasms arise. However, as Boveri already remarked in 1914, a major problem in the study of carcinogenesis is that it is impossible to identify a neoplasm "in statu nascendi".[13] Consequently, researchers postulate hypothetical narratives of what may have happened in the transition from normalcy to cancer.

Alternative theories of carcinogenesis were proposed based on competing premises. Some centered at the cellular level of biological organization and viewed cancer as a problem of cell proliferation or cell differentiation. Others, locked on the tissue level of biological organization, saw cancer as a problem akin to histogenesis.[14] Among many of the former, gross chromosome alterations and somatic mutations observed in advanced neoplasms were considered to be the causes of carcinogenesis.[15] Others interpreted these alterations to be just epiphenomena and considered carcinogenesis as an epigenetic process[16–18] (See Ref. 6, pp 99–111). These varied theories of carcinogenesis coexisted for most of the twentieth century. The methodological emphasis on molecular biological approaches initiated in the 1950s and 60s, plus the discovery of oncogenes in the 1970s, shifted this balance toward the acceptance of the SMT as the mainstream narrative of how neoplasms develop.

The somatic mutation theory

Internal inconsistencies and difficulties

From the SMT perspective, neoplasia is a cell-centered problem and, thus, the aim of cancer research was to uncover how a normal cell becomes a cancer cell. When Boveri introduced the first version of the SMT in 1914, it was believed that, in order to change the phenotype of a cell, its genotype had to be changed. Boveri assumed that cellular differentiation during embryogenesis was due to the unequal segregation of genetic material during cell division, a concept that was later abandoned because of the demonstrated genomic equivalence among somatic cells in adult organisms. However, the former concept was retained within the SMT due to (1) the existence of neoplasms transmitted by the germline (we will address this phenomenon below as "inborn errors of development") and (2) the observation that animals exposed to mutagens often developed neoplasms.

In the 1960s, genes (hitherto considered abstract, operational entities) were finally transformed into material, specific DNA sequences.[19] Molecular biologists concluded that biology was at last being reduced to chemistry. Consequently, describing chemical alterations in the genetic material became a more appealing approach to carcinogenesis than searching for messy, difficult-to-define interactions among cells and tissues.

The discovery that oncogenes were mutated versions of normal cellular genes led to the conceptualization of the cancer problem as that of gain-of-function mutations in genes that control cell proliferation and the cell cycle. Most of this research was conducted using in vitro models, such as primary cultures and established cell lines. Organismic phenomena were purportedly reduced to cellular phenomena. Neoplasms were reduced to a transformed cell and carcinogenesis was reduced to enhanced proliferation of cells in a dish. Verification of the tumorigenic potential of "transformed cells" was occasionally done by injecting millions of these "transformed cells" into the subcutaneous tissue of syngeneic animals and nude mice.

Soon after these one-step transformations were reported, amid much optimism that the phenomenon of carcinogenesis could at last be understood, the first critical voices noticed that carcinogenesis in animals, including humans, was a long process and, hence, something was missing in the models.[20] For example, infection with the Rous-sarcoma virus resulted in the transformation of chicken cells, an effect attributed to the *sarc* oncogene.[21] While the injection of Rous sarcoma viruses into chickens resulted in the integration of the *sarc* oncogene in all tissues, tumors only developed in places where wounds were inflicted.[22] In addition, the transformation of mouse fibroblasts by a single oncogene was attributed to the fact that the cells used as a model were not normal[10] because normal mouse fibroblasts were not transformed upon transfection with a single oncogene. At least two oncogenes were required.[23] In addition, according to Hahn and Weinberg, "attempts to transform primary human cells with combinations of oncogenes failed unless chemical or physical agents or stringent selection for rare immortalized variants was used".[10] This was attributed to a need for multiple additional mutations. If this were the case, then the dominant, gain-of-function effect attributed to the oncogene did not fulfill the original claims.

The study of heritable cancers, however, pointed in another direction. The gene alterations found were mostly deletions and cancer was therefore inherited when the genes were rendered inactive.[24] Retinoblastomas appeared to represent this type of tumor. The discovery of the *Rb* pathway allowed an explanation of transformation by means of SV40, a DNA virus that, unlike retroviruses, did not contain oncogenes. The large SV40 antigen interfered with the activity of *Rb*. Later on, it was reported that the small t protein of SV40 was necessary to achieve a tumorigenic state. This protein disturbed the activity of protein phosphatase 2A, which acts on a multitude of substrates. Mutations in subunits of this enzyme have been associated with cancers; however, these mutations have not illuminated the role of this enzyme in carcinogenesis.[10]

It was believed that, in the genesis of retinoblastomas in humans, in addition to the germline deletion, a second mutational event in the normal allele was sufficient to determine the neoplastic transformation of the retina (the two-hit hypothesis).[25] However, hemizygosity of the *Rb* gene in mice did not predispose animals to the disease, and *Rb*-deficient retinal cells underwent apoptosis in chimeras. Only the inactivation of Rb and p107 resulted in the development of retinoblastomas; yet, not all chimeric retinas in $Rb^{-/-}$ $p107^{-/-}$ mice developed tumors. Hence, additional events (mutational or not) appeared to be necessary for tumor development.[26] This and other examples of lack of fit led the supporters of the SMT to claim that mice may not be good models for human carcinogenesis after all.[27]

Among other familial cancers, colorectal cancer has probably yielded the most support for the SMT. About 15%

of these cancers occur in dominantly inherited patterns. In one of its forms, familial adenomatous polyposis, there is a deletion that, in most cases, results in a C-terminal truncated gene product in one of the two adenomatous polyposis coli (APC) genes. This disease results in the development of hundreds or even thousands of polyps between the second and third decade of life. However, inheritance of this mutated gene does not determine whether the carrier will always develop a cancer. For cancer to materialize, according to the SMT, other mutations have to occur. Yet, the same DNA lesion does not result in similar phenotypes. In addition, APC mutations are not absolutely required, since 15% of the carcinomas apparently express the full-length *APC* product. The function of the *APC* gene, which is expressed in many tissues, is unknown. Clues to the downstream effects of its inactivation were provided by the proteins that are recognized by the missing sequence in familial adenomatous polyposis. *APC* is expressed in the basolateral aspect of epithelial luminal cells. The C terminus binds to the human homolog of the *Drosophila* tumor suppressor gene discs large (*DLG*)[28] and to EB-1, a protein of unknown function.[29] The central portion of APC binds β-catenin, a protein that has at least two roles.[30] One is related to cell–cell adhesion through binding to cadherin and the other is signal transduction (*wnt* pathway). This suggests at least two ways through which APC inactivation may affect cellular processes connecting a cell with its surroundings. Rather than pointing directly to the control of cell cycle or cell proliferation, as expected from the tenets of the SMT, they point to the relation of the affected cell with its neighbors, the subject of the competing *tissue organization field theory* of carcinogenesis (TOFT), (see Ref 6, pp 91–143 and narrative below).

Other mutations, such as inactivation of *p53*, the "gatekeeper of the genome",[31] are also frequently observed in colorectal carcinomas. However, patients with germline mutations of *p53* do not develop colorectal carcinomas. Mutations in *RAS* frequently appear during progression of colorectal cancer; nevertheless, *RAS* mutations in the absence of APC alterations do not lead to the neoplastic state. Yet these mutations are found in foci of proliferating cells. The problems posed by these findings led Kinzler and Vogelstein to ponder, "...it is not simply the accumulation of mutations, but rather it is also their order, that determines the propensity for neoplasia, and that only a subset of the genes which can affect cell growth can actually initiate the neoplastic process".[32] However, these cumulative findings are not supportive of the main notion imbedded in the SMT, that is, that genotype drives the phenotype through alterations of the ability of cells to proliferate.

The question of how many DNA mutations a single normal cell has to withstand to become a cancer cell has been a major concern, since the normal rate of mutations in somatic cells could not account for the number found in neoplasms.[33] The study of hereditary non-polyposis colorectal cancer (HNPCC)

that harbors mutations in mismatch repair genes provided an example of hypermutability in colorectal cancer. However, these tumors represent only a small percentage of colorectal cancer; 85% do not show this high mutation rate, but have instead a propensity to show aneuploidy. The absence of aneuploidy in HNPCC (cells are usually diploid in these tumors) challenges the long-held idea that these rearrangements were the consequence of excessive cell divisions. Some HNPCC patients were found to undergo elevated rates of mutations in their phenotypically normal cells, which were explained by a deficit in mismatch repair activity.[34] Remarkably, these patients do not have increased rates of cancer in tissues other than the colon. This is consistent with experiments in mice whereby targeted disruption of these genes does not result in high cancer incidence.[35]

Proponents of the SMT assume that more research along the current lines will provide data that will reconcile the present paradoxes and reveal general unifying rules. However, the search for those unifying rules appears thwarted by reports claiming that " . . . oncogenes and tumor suppressor genes are important not only for cell proliferation but also for cell fate determination (differentiation, senescence, and apoptosis), their effects often depending on the type of cell in which they are expressed. Thus, overexpression of a given oncogene can enhance growth in one cell type but inhibit growth or induce apoptosis in another".[36] This statement about the context-dependence of oncogene activity contradicts the original concept, namely, that oncogenes are dominant gain-of-function mutants of normal genes that should cause increased cell proliferation.

Criticism from without: is the default state of metazoan cells proliferative quiescence?

As noted above, the second premise adopted by those who favor the SMT is that the default state of cell proliferation in metazoa is quiescence.[5] By default state, we mean the state under which cells are found when they are freed from any active control. We consider this an implicit premise because it is seldom acknowledged. Since growth factors are invoked as the levers that putatively stimulate proliferation, quiescence implicitly becomes the default state of these cells (See Ref 6, pp 1–13).

Why should we care about the default state? We have previously addressed this issue both experimentally as well as epistemologically[37–40] (see Ref. 6, pp 1–30). From a practical point of view, it matters because in adopting the premise that the default state is quiescence, researchers become committed to favoring the notion of positive control of cell proliferation and, thus, to the search for growth factors. If, instead, researchers adopt the opposite premise, namely that the default state of cells is proliferation, they would introduce the notion of negative control of cell proliferation and would search for inhibitors. But why do we have to choose among

these postulates when dealing with carcinogenesis, or with developmental biology at large, for that matter? The default state of unicellular organisms (both prokaryotes and eukaryotes) and metaphyta is widely accepted to be proliferation. However, not much discussion has been devoted to the default state of cells in metazoa. In fact, it has been assumed all along that the default state of metazoan cells is quiescence. No explanations or data are given to support such a drastic evolutionary change.[5,41] Thus, researchers are left to choose between these exclusive postulates.

To resolve this conundrum, the choice need not be arbitrary. From an evolutionary perspective, the generation of multicellular organisms from unicellular eukaryotes involved the conservation of pre-existing levels of organization. The built-in capacity for self-replication by cells within a multicellular organism must have remained unaltered and hence, their default state conserved. The following arguments support this concept.

(1) Multicellular organisms develop from a single cell—the zygote—that in many species initiates development outside the parental organism, and therefore proliferates without exogenous intervention of putative growth factors.

(2) There is almost complete homology between the replication machinery of yeast and human cells, suggesting that the process remained constant throughout evolution. Unicellular organisms multiply as long as nutrients are available. With the advent of multicellularity, the coordination of the proliferative activity of each lineage making the different tissues of the organism required the emergence of negative controls that impose a quiescent state upon cells. Once these cells are freed from organismal restraints, they reacquire their default state and proliferate (see Ref. 6, pp 41–77 and 134–143).

(3) The few studies performed to experimentally address the nature of the default state suggest that proliferation is the default state of metazoan cells.[37,40,42]

How could proliferation as the common default state have been ignored? At the beginning of the 20th century, experimentalists resorted in earnest to growing cells in culture conditions to resolve problems raised by the complexity of organisms. From an experimental perspective, evidence that the default state of unicellular organisms and metaphyta is proliferation is not hard to find, since a multitude of unicellular organisms and plant cells can be propagated in a simple nutrient mixture.

The problem posed by cells from metazoa is that, for the most part, they require a complex medium containing macromolecules. Only a few cell lines are easily propagated in defined medium. It may be argued that the difficulty found by early practitioners in getting metazoan cells to propagate in

glass flasks created the misconception that they had to be "stimulated" by adding singly, or in combination, a variety of supplements (i.e. embryo extracts or serum) to the culture medium. Under these operational circumstances, these supplements became generically known as "growth factors". It should be mentioned that this was the operational definition of any substance that improved the propagation of bacteria as well; some pathogens absolutely required macromolecules in order to propagate. Later, the requirement of these "growth factors" for the propagation of metazoan cells was construed to mean that their default state was quiescence and that serum contained specific signals that induced cell proliferation. The term "growth factors" then acquired a narrow, regulatory meaning. The fact that, in the absence of these macromolecules, the metazoan cells were not quiescent but dead must have been overlooked.

Despite granting that this was the dawn of a novel approach to experimental biology, the patent lack of fit with evolutionary theory should have caught the attention of rigorously trained biologists (see Ref. 6, pp 14–30). Through ontological economy or by application of the parsimony principle (Ockham's razor), no new entity should be needlessly postulated. Experimentally accumulated evidence supported the notion that cells that did not proliferate much in the intact animal organism, e.g. fibroblasts, did so soon after being transferred to a synthetic, serumless culture medium in a glass or plastic dish. The failure to permanently maintain this dominant proliferative condition may have also mislead researchers and uncommitted observers into favoring the need to add operational growth factors (usually polypeptides) to the culture medium. Recent data on the role of these putative "growth factors" supports the notion that they are either "survival" factors,[43,44] or, as in the case of hormones, that they act indirectly by neutralizing the effect of specific inhibitors.[40] The literature on genetically engineered knockout mice also shows that the so-called growth factors play important roles in cell fate, migration, and a myriad of developmental processes, but they do not specifically act on the process they were originally supposed to control, i.e., to induce quiescent (G_0/G_1) cells to enter the cycle.

For the last two decades, our research program has been based on the premise that the default state of all cells was proliferation.[39,45,46] Recently, Henry Harris, a pioneer of somatic cell genetics, concurred with this notion.[47] Our reinterpretation of a concept so central to life is not an academic issue. Its implications on the understanding of carcinogenesis cannot be overemphasized, especially in the context of the TOFT (see below).

A cellular approach to differentiation: somatic cell hybrids

Harris considered carcinogenesis to be a cellular phenomenon, whereby loss-of-function changes in the DNA deter-mined the cancer phenotype. He observed that the behavior of cancer cells was "normalized" by hybridization with normal cells; this resulted in the lack of tumor formation when the hybrid cells were injected subcutaneously into nude mice. These data are consistent with the existence of suppressor genes and inconsistent with that of oncogenes. In his own words, "As things now stand, it appears that the key cellular events determining malignancy are heritable losses of function, and, in particular, loss of the ability to complete specific patterns of differentiation. This may well be true not only for genetic lesions involving tumor suppressor genes, where the evidence is in some cases compelling, but also for mutated oncogenes. The two great peaks that somatic cell geneticists have long been attempting to scale, cancer and differentiation, seem to have merged into one".[48] In order to explore the mechanisms underlying the suppression of the neoplastic phenotype in hybrids between normal and neoplastic cells, Harris transfected neoplastic cells with cDNAs expressing proteins such as keratins, which are markers of terminal differentiation of keratinocytes, in order to force cells to differentiate and thus behave normally. This particular strategy did not produce the anticipated results.[49] Harris also disrupted the pattern of fibronectin expression by the introduction of antisense fibronectin constructs into non-tumorigenic hybrid cells. The "malignant" phenotype re-appeared in the cells in which the antisense construct resulted in reduction of fibronectin synthesis.[50]. Hence, in his view, carcinogenesis does not require acquisition of a new function, but rather the disruption of the pattern of cellular differentiation.[47]

"Normalization" of cancer cells in an organismal, tri-dimensional context

When early embryos were transplanted into ectopic places (e.g. the kidney capsule or the peritoneal cavity), they behaved like malignant neoplasms called teratocarcinomas. Conversely, teratocarcinoma cells injected into early embryos (blastocyst stage) generated normal tissues and organs. In fact, those cancer cells became gametes (oocytes and sperm cells), which in turn generated normal progeny. Thus, embryonal cells produced neoplasms when misplaced in adult tissues and reverted to normalcy when placed into an early embryo.[51] In addition, when nuclei from Lucke's frog renal carcinoma cells were transplanted into enucleated and activated ova, they developed and reached the swimming tadpole stage.[52] Additionally, transplantation of tissues from these tadpoles into normal recipients generated normal tissues that were indistinguishable from those of the host.[53] These data challenged the view that cancer was caused by DNA mutations, since the neoplastic phenotype could be normalized at a frequency much higher than was needed to revert a DNA mutation back to the wild type. Hence, the dictum "once a cancer cell, always a cancer cell" was invalidated and

the data instead suggested an epigenetic control of the expression of neoplastic phenotypes.[17]

Although these experiments clearly showed the reversibility of the neoplastic phenotype, and hence are inconsistent with the SMT, they did not address the issue of how neoplasms arise. In this regard, the relevant question that needs to be asked is: at what level of biological complexity does carcinogenesis occur?

Carcinogenesis and biological organization

Cancer occupies multiple levels of biological organization[46] (see Ref 6, pp 91–143). Within this perspective, determining at which of these levels carcinogenesis occurs is controversial. To illustrate this concept, we introduce the image of a metaphorical bookshelf. In this bookshelf, each separate volume would deal with and represent a different level of complexity. Each volume would be lying side by side with others addressing "higher" or "lower" levels. The information contained in each volume may have only a limited relatedness, or none at all, with that presented in another volume of the collection. A historic example will be representative of this type of relationship at the population level of organization. Over a half-century ago, epidemiologists and public health officials were able to design and promote effective preventive campaigns for a good number of cancers. Specifically, reducing tobacco consumption in order to lower lung cancer incidence did not require that those epidemiologists and public health professionals of yesteryears knew much about DNA replication, gene expression, signal transduction pathways, or epithelium-stroma interactions. A comparable case could apply to the design of a vaccination campaign against the hepatitis B virus to reduce the incidence of hepatocellular carcinomas, or to the eradication of schistosomiasis to diminish the number of bladder adenocarcinoma cases.

Returning to our metaphorical bookshelf, another volume should be dedicated to cancer at the organismal level, the equivalent to cancer disease management. This is the level at which a patient interacts with his/her physician. These protagonists exchange information about the symptoms and signs of the cancer syndrome. After the initial contact with the patient, the clinician makes a preliminary diagnosis of the disease through physical examination and by reading the results from a battery of tests that he/she has ordered. Later on, if the diagnosis is confirmed, an interactive managerial relationship is established between the patient and his/her treatment group.

However, another physician, the pathologist, is the one who makes the final, definitive diagnosis when he/she "reads" a biopsy of the suspected neoplastic tissue through an uncomplicated light microscope. Thus, by this objective criterion, a separate volume in our metaphorical cancer bookshelf should be dedicated to carcinogenesis at the level where it is identified, i.e. at the tissue level of biological complexity. We

postulate that this is the level at which carcinogenesis takes place (see below).

By extending the metaphorical argument of the cancer bookshelf, we conclude that a volume dealing with cancer at the subcellular level of organization should, at best, be moved to a library shelf where generic subcellular and biochemical topics are placed, or at worst, be considered apocryphal. We hasten to add that the effects of carcinogens on subcellular structures and organelles (including genomic mutations), while variably deleterious to each and every cell in the host, are not viewed as directly responsible for the development of neoplasias.

Thus, a rationale that favors discarding the SMT is predicated on the grounds that its niche is at the subcellular level of biological complexity, a level that appears as irrelevant to carcinogenesis.[46] This conclusion does not imply that the gigantic effort invested in describing changes at the gene level (gene mutations, methylation patterns, gene expression, etc.) and/or that of the cellular organelles (endoplasmic reticulum, Golgi apparatus, mitochondria, etc.) was fruitless. These data, frequently obtained while using human and rodent tumor cells in culture conditions, have significantly increased our understanding of normal intracellular processes. We posit, however, that these features are not unique or specific to the cancer state, that they are instead part of the flexible set of phenotypic variations with which cells are normally endowed. Hence, it would be understandable that they have fallen short of providing an explanation for carcinogenesis. To the contrary, an examination of a biopsy by a competent specialist would be enough to discriminate between a normal histoarchitectural pattern and that of a neoplasm.

The tissue organization field theory of carcinogenesis

Organicism and developmental mechanics as sources of TOFT

Reconstructing the history of concepts that led to the TOFT is beyond the scope of this review. Suffice it to say that at the end of the 19th century, Boll proposed that cancer resulted from inductive interactions between tissues, Cohnheim suggested that cancer originated in embryonic residues, and Ribbert postulated that epithelial cells do not possess special proliferative powers, but that their proliferation results from being freed from the restrictions imposed by normal tissue organization.[54,55] Yet, the introduction of the morphogenetic field concept was a central event in the genesis of the TOFT (see Ref. 6, pp 91–143). "Fields of organization" or "morphogenetic fields"[56] were defined as "a collection of cells by whose interactions a particular organ formed".[57] The morphogenetic field became the basic paradigm of embryology. In the 1930s, Needham[58] and Waddington[59] speculated that neoplastic development resulted from alterations of the normal

interactions that occur in those morphogenetic fields. In other words, carcinogens, as teratogens (i.e. agents that interfere with normal embryonic development), would disrupt the normal dynamic interaction of neighboring cells and tissues both during early development and throughout adulthood. This concept was updated by Rubin 50 years later.[60]

Organicism has provided the philosophical bases for the study of embryology's modern beginnings.[61] Biologists of the organicist persuasion ask questions about self-organization, cell–cell interactions, tissue–tissue interactions, and organogenesis. They posit that the organism is the zygote that organizes itself into a newborn and beyond. By virtue of being an open system, the organism utilizes resources from both the external (environment) and the internal (gene products and other chemicals synthesized by the organism) worlds. As the reductionistic and genetic determinist view became dominant in biology, the organicists continued their studies of self-organization. Their explanations are operational and are made in terms of cell–cell and tissue–tissue interactions. In contrast, reductionist explanations are made in terms construed as material entities such as genes and their products. From this perspective, histogenesis and organogenesis were supposed to be reduced to the phenomenon of differential gene expression, which was thought to be similar in bacteria and in multicellular organisms. As stated by Jacques Monod "what's true for *E. coli* is true for an elephant".[62] For a long period, the mechanicist rhetoric of geneticists won the day.

From a reductionistic perspective, tissues became collections of independent cells and explanations of carcinogenesis were sought primarily at the cellular, subcellular and molecular levels of organization. To explain differentiation and epigenesis, the morphogenetic field was overcome by the operon, a group of genes controlled by the same regulatory gene. In fact, the morphogenetic field hypothesis was not disproved, it was just forgotten.[57] Only when morphogen gradients were visualized toward the end of the 1990s did developmental biology resuscitate this old concept so central to its previous success.[63] Morphogens are diffusible substances that "determine" the differentiation that cells "perceiving" this information will undergo (http://www.books.md/M/dic/morphogen.php).

As briefly noted above, despite the dominance of the reductionistic program, a few research groups studied the expression of the neoplastic phenotype in a developmental context such as in teratocarcinomas and Lucke's tumors, while others addressed the role of tri-dimensional organization and extracellular matrix.[64]

Premises and supporting evidence

The TOFT is based on two main premises: (1) that carcinogens act initially by disrupting the normal interactions that take place among cells in the stroma and parenchyma of an organ,[46,58,59,65], and (2) that proliferation is the default state

of all cells (see Ref 6, pp 1–30). During embryonal and fetal development, epithelium and the subjacent stroma exert instructive influences over each other. These morphogenetic fields remain operational during adulthood.[60] The disruption of these interactions by carcinogens results in a lessening of the cells' ability to "read" their positional and historical background. This, in turn, allows the epithelial cells to exercise their constitutive property to proliferate (hyperplasia). Next, the tissue organizational pattern would become disrupted (dysplasia) or would even adopt a different tissue type (metaplasia). The pattern of progression to carcinoma in situ may not always exactly follow this sequence.[66] However, this pattern prevails in carcinomas and adenocarcinomas, which represent the substantial majority of human neoplasms.

Central to this dynamic process is its reversibility.[66] The neoplastic phenotype can be experimentally reversed through cell–cell interactions as demonstrated by embryonal carcinoma cells injected into blastocysts,[67] hepatocellular carcinoma cells injected into normal livers,[68] or modification of the extracellular matrix components.[69,70] Hence, the cancer phenotype is an adaptive, emergent phenomenon occurring at the tissue level of organization and is susceptible to being normalized. Of course, if the irritative action of the carcinogen persists, or if the histoarchitecture has been severely compromised, eventually a full neoplastic state evolves, thus diminishing the chances of returning to the status quo ante (see Ref. 6, pp 91–143).

Using a theory-neutral experimental strategy, we recently collected data on rat mammary carcinogenesis. We observed that the recombination of stroma exposed to a carcinogen with normal epithelial cells resulted in neoplasms. The reverse combination did not. This observation suggests that the stroma, rather than the epithelium, is the target of the carcinogen.[71] These results challenge the validity of the SMT, while buttressing the TOFT.

Sporadic versus hereditary cancers

From our perspective, hereditary cancers[24] should be considered as *inborn errors of development*. Analogous to inborn errors of metabolism that were extensively described during the second half of the twentieth century,[72] these cancers represent syndromes that involve the appearing of uni or multilocular tumors at different times during development. For instance, these syndromes may appear shortly after birth as in retinoblastoma,[73] after puberty or in early adulthood like in multiple endocrine cancers,[74] or prior to the age of incidence for the non-familial form in breast cancers due to BRCA1 and BRCA2 gene mutations,[75] and in colorectal cancers due to APC mutations. The distinction between sporadic and hereditary cancers is intended to separate two sets of tumors that have a distinct etiology (epigenetic versus genetic, respectively) but share a common pathogenesis (tissue architecture disruption).

Are there ways to reconcile the SMT and the TOFT?

These two theories are not compatible in principle. While one centers on "one renegade cell", as asserted by R.A. Weinberg[4] and views cancer as a cell-based disease involving unregulated cell proliferation, the other focuses on a "society of cells"[6] and views cancer as a problem of tissue organization. However, this does not mean that the data gathered from experiments based on the SMT cannot be interpreted in the light and context of the TOFT. The polyps in patients who are hemizygous for a defective *APC* and the displasias appearing prior to neoplasia in retinoblastoma and in the lethal giant larva mutant in *Drosophila* are all tissue organization alterations. In the case of inactivated *APC*, one may even hint at the mechanisms that may be involved, since, as mentioned above, *APC* binds to β-catenin, which in turn binds to cell adhesion molecules called cadherins.[76] *APC* also binds to the human homologue of *Drosophila discs large (hDdl)*, which is also involved in cell–cell adhesion through septate junctions.[77] Deletions of this gene in flies result in the loosening of cell–cell contacts, abnormal morphology of the imaginal discs, and neoplastic development.[78]

Altered communication among cells is at the core of the TOFT. From this perspective, one would study how specific alterations in *APC*, catenins, cadherins and *hDdl* affect the development of the intestinal crypt and give rise to polyps. Instead, the SMT-based research effort centers on the role of β-catenin as a transcription factor and looks at the epithelial cell nucleus (the transcriptional machinery) for putative alterations in the control of cell proliferation, cell cycle and apoptosis. It is thus theoretically conceivable that spontaneous gene mutations causing altered cell–cell communication may lead to carcinogenesis; the biological effects of these mutations, however, would only become apparent at the tissue level of organization.

A different problem is revealed by the study of the *lethal giant larva-2 (lgl-2)* gene in *Drosophila*. A deletion in this gene is responsible for the development of neuroblastomas in homozygote flies. This gene is expressed when the embryo is a synctium and is never expressed in the cell type that becomes cancerous when the gene is defective, i.e., neuroblasts. As the nervous tissue develops in the mutant *Drosophila* larva, it appears less organized than in its normal counterpart.[79] Thus, the gene deletion somehow affects tissue organization several steps downstream after it failed to be expressed much earlier. Hence, even finding the mutated gene and showing its causal role in carcinogenesis has fallen short of explaining the cancer phenotype.

Ectopic expression of normal genes in transgenic mice results in neoplastic development as observed by Sternlicht et al,[80] who reported that manipulations of the microenvironment, such as overexpression of stromelysin 1, may result in carcinogenesis. This matrix metalloproteinase would alter cell–cell and cell–extracellular matrix interactions. These alterations, in turn, would promote the neoplastic transformation of the mammary gland. Moreover, administration of proteinase inhibitors suppressed the carcinogenic process that ensued when the stromelysin-1 transgene was expressed.[80] Interestingly, the resulting neoplasms displayed DNA losses in chromosomes 4 and 7, and those showing epithelial–mesenchymal transitions displayed DNA gains. Hence, alterations in tissue architecture can and do induce neoplasms, and those neoplasms, like the sporadic ones, may end up showing aneuploidy. As Prehn remarked, "...it may be more correct to say that cancers beget mutations than it is to say that mutations beget cancers".[16]

In sum, genes causing inborn errors of development and cancer could easily be incorporated into the TOFT, but the questions asked about the role of these genes would be different from those formulated by the SMT. While the former looks at cell interactions in a tissue-based, developmental context, the latter looks at the cell as a quasi-autonomous entity, governed from the inside by its genes. As put by L. Moss: "To heirs of nineteenth century holism ('organicism'- is the materialist, contemporary version of holism-author's note), autonomy was understood in terms of 'totipotency', the possession by the cell of the potential of the whole. The autonomy of the cell understood this way is then the precondition for either normal or aberrant growth and a prior guarantee of neither. What determines which way it will go, normal or aberrant, is not its internal features but the subsequent history of its interactions" (see Ref 81, p 129).

Back to the beginning: a historical and philosophical perspective

For four centuries, choices between competing postulates, hypothesis testing and falsification have been central to the long, successful tradition of science. Only after a rigorous weeding-out process is a synthesis possible. Through this synthesis, contradictions are resolved and both spurious "facts" and wrong premises are recognized and dismissed. A misguided, premature synthesis may lead to a confusing state of affairs where, if results do not fit one hypothesis, they may fit its opposite; in other words, nothing is rejected and everything is explained by the piling up of ad hoc explanations. This attitude contrasts with the objectives of science as described by Ayala, namely: "(1) science seeks to organize knowledge in a systematic way, endeavoring patterns of relationship between phenomena and processes; (2) science strives to provide explanations for the occurrence of events; and finally, (3) science proposes explanatory hypotheses that must be testable, that is, accessible to the possibility of rejection or falsification".[82]

When assessing the state of the art in carcinogenesis at the beginning of the 21st century, we are reminded of a similar evaluation done in 1926 about the state of the art in

embryology. H.S. Jennings recalled that embryologists often did similar experiments and arrived at different conclusions. "All the conflicting reports were correct. The situation was that of the Gilbertian comic opera chorus, 'For you are right, and I am right, and he is right and all is right'".[83] Maienschein's historical analysis shows that, at the root, those were issues of epistemology; researchers were disagreeing not only about the biological phenomenon, but also about how it should be studied.[84] A rigorous epistemologic foundation helps guide experimental design, the gathering of data, and the interpretation for or against a given hypothesis.[85–87] Hence, it is not disruptive, but actually productive, that alternative views coexist before the body of evidence gathered allows for a synthesis and/or the rejection of the wrong concepts and hypotheses.

As we have analyzed above, the emergence of conflicting data within the SMT did not result in the rejection of premises and hypotheses. For example, an oncogene could be "dominant" and express a gain of function with respect to the non-mutated homologue, and its biological effect could be contextual at the same time. That is, a mutation that should have produced uncontrolled cell proliferation resulted in cell death or arrest of cell proliferation. Again, ad hoc explanations were proposed to resolve conflicting evidence, leading to a situation whereby any possible conclusion is valid because no alternative concept is ever disproved and abandoned. The lack of fit is attributed to the unfathomable complexity of nature/biology.[88] In short, something can be anything and its opposite.

In this atmosphere, an attempt to blend "tissue-based" cancer research into the oncogene theory has been proposed. Namely, data showing that extracellular matrix and tissue architecture can normalize the behavior of cancer cells[89] are re-interpreted by adherents to the SMT as important steps towards understanding the mechanisms that determine how "...cancer genes perturb the biological interactions of individual cells with their immediate surroundings".[90] Hence, for these committed supporters of the SMT, the problem of how extracellular matrix controls cell phenotypes becomes at best a quest to unravel how oncogenes affect interactions between mutated and normal cells.

The philosopher L. Moss has put forward the argument that most of the problems inherent to the SMT are due to the amalgamation of the Mendelian gene (as used in transmission genetics to trace the inheritance of a given character) with the molecular gene (a DNA sequence) and to the adoption of a preformationistic view in the long and still ongoing debate between epigeneticists and preformationists (see Ref. 81, pp 183–198). Indeed a substantial literature, both biological and epistemological, clearly shows that the Mendelian gene was not reduced to the DNA "gene" and that the relationship between the two is rendered ambiguous because of splicing (one gene–many possible transcripts) as well as by the

classical properties of pleiotropism (one gene–diverse effects) and polyphenism (one genotype–multiple phenotypes).

Regarding the preformationism/epigenesis argument in embryology, the 18th century homunculus that determined morphogenesis in the embryo morphed into a genetic program in the middle of the 20th century. The modern view about epigenesis is instead that the embryo constructs itself, using not only the proteins and RNA coded in the genome, but all sorts of environmental resources. According to Moss: "The critical decisions made at the nodal points of organismic development and organismic life are not made by a prewritten script, program, or master plan but rather are made on the spot by an ad hoc committee" (see Ref. 81, p.186).

Conclusions

During the last decades, developmental biology benefited from many conceptual and methodological advances involving the role of interactions among extracellular matrix, cells and tissues in morphogenesis. The application of the morphogenetic field concept to cancer research has revealed that the neoplastic phenotype can be reversed when cells from a neoplasm are placed in a normal environment.[6,46,67–69,81] The normal interaction among tissues during development may be disrupted by a variety of physical, chemical and biological agents resulting in malformations. Similarly, disruption of these same interactions during adulthood may result in neoplasia.

From a methodological standpoint, those favoring the premises of the SMT adopted an in vitro, two-dimensional approach involving a single cell type to study carcinogenesis. Instead, the TOFT favors adopting the methods and strategies used by developmental biologists to study histogenesis and organogenesis, including the use of tissue recombination in animals and, when warranted, a three-dimensional model where different, interacting cell types in culture evolve into a series of changes that mimic what happens in the complex environment of tissues in situ.

From an epistemological viewpoint, the TOFT removes the gene from the driver's seat (genetic determinism), and brings the organism and its ability to self-organize as the conceptual focus (organicism). This parallels the position of Smithers who, over four decades ago, compared the merits of cytologism and an organismal view of carcinogenesis.[14]

Historically, replacing an old paradigm with a new one is a drawn-out enterprise.[91] Science, being a creation of the human intellect, becomes subject to the vagaries of social activities where the participants have interests that transcend the objective value of the competing paradigms.[92] In the modern era, awareness of these vagaries on the part of governmental and private funding agencies may accelerate and productively stir these changes for the benefit of both the public at large and that of the research community. In the meantime, much will be accomplished when cancer research

rejoins the long and successful tradition of discarding premises and rejecting hypotheses.

Note added in proof

Almost three decades after B. Mintz's group showed the "normalization" of teratocarcinoma cells implanted into mouse blastocysts, Hochedlinger et al (Reprogramming of a melanoma genome by nuclear transplantation, Hochedlinger K., Blelloch R., Brennan C., Yamada Y., Kim M., Chin L. and Jaenisch R. 2004 Genes & Development, 18:1875–1885) now demonstrate the pluripotentiality of the nuclei of mouse melanoma cells. In their elegant experimental approach, these authors extend further the notion of the potential reversibility of the cancer phenotype under the influence of a normal multicellular environment.

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STROMAL REGULATION OF NEOPLASTIC DEVELOPMENT:

AGE-DEPENDENT NORMALIZATION OF NEOPLASTIC

MAMMARY CELLS BY MAMMARY STROMA

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Abstract

We report that mammary gland stroma from mature and multiparous rats prevent neoplastic development and encourage normal ductal growth of grafted epithelial cancer cells. Fifty thousand epithelial cancer cells were injected into the cleared fat pads of virgin hosts at ages 24, 52, 80 and 150, and into hosts that had undergone two cycles of pregnancy and lactation. Tumor incidence was measured six months later. The tumor incidence was 75%, 100%, 50%, and 18.2% when epithelial tumor cells were inoculated into 24, 52, 80 and 150 day-old virgin rats, respectively, and 0% in the twice-parous animals. Most remarkably, these neoplastic cells also formed normal ducts in all hosts. This tumor development pattern suggests a parallel to the phenomenon of age- and reproductive state-dependent susceptibility and resistance to chemical carcinogens. As susceptibility to carcinogenesis decreases, the ability of the stroma to reprogram neoplastic epithelial cells increases. Thus, the neoplastic phenotype is context-dependent and it therefore offers the intriguing possibility that the process of carcinogenesis is amenable to normalization or "cure" once components of the mechanisms of stroma-mediated normalization are elucidated and manipulated.

Introduction

During early development, the mesenchyme plays inductive and permissive roles in epithelial morphogenesis, differentiation and proliferation. These events have been observed in experimental models both *in vitro* [1,2] and *in vivo* [3]. During adult life, the stroma play a comparable role in the maintenance of the structure and function of epithelia. An equally prominent role for the stroma has been verified experimentally during the process of carcinogenesis in several organs [4-7].

Using a tissue recombination model, we and others recently observed that the stroma plays a crucial role in mammary gland carcinogenesis [7]. Specifically, rat mammary adenocarcinomas occurred only when the mammary stroma was exposed to the chemical carcinogen N-nitrosomethylurea (NMU), regardless of whether the epithelial cells were exposed as well. On the other hand, it has also been shown that carcinoma-associated stromal cells have the capacity to "transform" non-tumorigenic epithelial cells into neoplasms[8-10]. Altogether, these experimental observations support the concept that carcinogenesis and neoplasia are emergent, supracellular phenomena [11-13].

In a different but related context, the results obtained by Rivera and co-workers in the 1980s suggest another role for the stroma, namely, that of normalizing or reprogramming mammary cancer cells *in vivo*. Neoplastic epithelial cells and tissue fragments obtained from primary mammary tumors developed into secondary tumors upon inoculation into cleared mammary fat pads (CFPs) [14,15]. Insightfully, Rivera and co-workers observed that phenotypically normal ducts were also present in the hosts' CFPs in the recombinant

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tissues. However, this phenomenon was not investigated further, probably because it could not be explained within the context of the prevailing *somatic mutation theory* (SMT). The main assumption of the SMT is that neoplasms are the result of accumulated mutations in the DNA of an epithelial cell. After two decades of research highlighting the importance of the extracellular matrix and of stromal-epithelial interactions on the expression and suppression of neoplastic phenotypes, Rivera's observations can now be re-interpreted in the context of the *tissue organization field theory* (TOFT), which posits that carcinogenesis is a tissue-based process, akin to development gone awry [11].

One of the predictions of the TOFT is that carcinogenesis can potentially be reversed. This would occur when the normal tissue morphogenetic unit (stroma and epithelium) is re-established and the constitutive proliferative ability of epithelial cells is inhibited [12;16;17]. Experimentally, the reversal of neoplastic behavior has been accomplished repeatedly when neoplastic cells were placed within the normal tissues from which they originated. For instance, in a series of elegant experiments, Mintz and collaborators showed that teratocarcinoma cells injected into blastocysts became integrated into the normal tissues of the mosaic mice [18]. More recently, McCullough *et al.* observed that hepatocellular carcinoma cells formed aggressive tumors when injected subcutaneously but became integrated into the normal tissue when placed into the liver of syngeneic animals [19]. On the other hand, Weaver and collaborators have shown reversion of the malignant phenotype of breast cells *in vitro* by modifying the cell surface β1 and β4 integrins in a 3-dimensional basement membrane assay [20].

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Spontaneous regression has been reported in almost all types of human neoplasias [13;21]. Although only a few cases of spontaneous regression of breast cancer have been documented, rigorously conducted recent mammographic studies suggest that this phenomenon may be more common than previously thought [22-24].

Based on the above background information, we decided to further explore this subject using the rat mammary gland as an experimental model. Thus, we chose to test whether age and parity affects the ability of the stroma to support or repress tumor development. In order to test their potential to form either normal ducts or neoplasms, we transplanted neoplastic epithelial cells into CFPs of virgin rats of different ages and into animals that had completed two pregnancies (including lactation and involution). This report is part of an extended, detailed effort to map out the influences of the rat mammary stroma on carcinogenesis and tumor regression.

Materials and Methods

Animals

Wistar-Furth (WF) rats were purchased from Harlan (Indianapolis, IN) and housed in transparent plastic cages with food and water ad libitum. Animals were maintained on a 14:10 hours light:dark cycle and care was in accordance with the Guidelines for the Care and Use of Animals and the Tufts-New England Medical Center Institutional Animal Care and Use Committee.

Induction of mammary tumors

Virgin 52-day-old female rats were injected intraperitoneally with a single dose of 50 mg of NMU/kg body weight. Tumors were palpable beginning at 12 weeks after treatment. These tumors were designated donor tumors to distinguish them from those tumors derived from the inoculated neoplastic epithelial cells which were arbitrarily called secondary tumors.

Preparation of cells for transplantation

Cells were prepared according to the method described by Alston-Mills and Rivera with minor modifications [7;25]. Briefly, when tumors reached approximately 1.5 cm in diameter they were removed and placed in sterile phenol red-free Dulbecco's modified Eagle medium (DMEM). The tumors were minced and digested in phenol red-free DMEM containing 0.1% collagenase type 3 (Worthington, Lakewood, NJ) at 37°C for 2 hours while agitating. This digest was centrifuged and the pellet was then treated with 1.25% pronase (Calbiochem, San Diego, CA) for 5 minutes at 37°C with agitation. This cell suspension was filtered through a 530 μm-pore Nitex® filter (Sefar America, Kansas City, MO) and the filtrate was centrifuged at 100 xg for 3 minutes. Subsequent filtrations were performed using a 250 μm-pore filter, then a 10 μm-pore filter. The cells were counted with a Coulter Counter ZM and resuspended in DMEM.

Hosts for tumor cell transplantation

The mammary epithelium was surgically removed from the 4th and 5th right abdominal-inguinal mammary glands (CFP) of 10-day-old rats, according to procedures that were

originally outlined by DeOme *et al* [26] and done routinely in our lab. The left abdominal-inguinal mammary glands were left intact and considered internal controls. In each of the animals used in these experiments, the excised epithelium was whole-mounted and observed microscopically to assure that the ductal tree was removed in its entirety and that only a small portion of the fat pad remained attached to it. The host rats were separated into two groups: one of virgin females of 24, 52, 80 and 150 days of age, and another of twice-parous females. The twice-parous rats were bred starting at two months of age. In all these rats, the 4th CFP was used as the transplantation site.

Cell transplantation

Using a Hamilton syringe (Hamilton Co., Reno, NV), 5×10^4 cells contained in a 10 μl volume were injected into the right side CFP. Starting one month after the cell inoculation, all rats that received a cell transplant were palpated weekly. Animals were sacrificed when tumors reached 1 cm in diameter or 6 months after cell transplant, whichever occurred first. Animals were excluded from the analyses when no ductal epithelial outgrowths were found in the whole mounts ("no takes") or when they died as a result of surgical complications. The initial (i) and final (f) sample sizes at 6 months after the cell injection were as follows: age 24 i=9, f=8; age 52 i=9, f=7; age 80 i=11, f=10; age 150 i=11, f=11; twice-parous rats i=7, f=5.

Whole mounts and histology

Whole mounts were prepared following protocols described by the mammary.nih.gov website [27], and Thompson *et al.* [28]. The mammary glands were removed and spread on a

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75 x 50 x 1 mm glass slide (Fisher Scientific, Pittsburgh, PA), fixed overnight in 10% phosphate buffered formalin, dehydrated in 70%, 95% and 100% alcohols, cleared in toluene, rehydrated and stained with carmine alum. After staining, the whole mounts were dehydrated as described above, cleared in xylene, and bagged in Kpak® SealPak heat-seal pouches (Kpak Corp., Minneapolis, MN) in methyl salicylate. The whole mounts were analyzed under a stereomicroscope Stemi 2000 (Carls Zeiss, Munchen-Hallbergmoos, Germany) for detection of microscopic lesions. Tumors larger than 0.5 cm were removed before the whole mounts were prepared, separately fixed as described above, and paraffin-embedded for histological analysis. Microscopic lesions were removed and also embedded in paraffin for histological analysis. Images were captured with an AxioCam HR color digital camera (Carl Zeiss, Munchen-Hallbergmoos, Germany) attached to a stereomicroscope.

DNA extraction and analysis of Ha-*ras*-1 gene mutation

DNA was extracted from the donor tumors, the secondary neoplasms (both palpable tumors and microscopic lesions), and the normal outgrowths using a DNeasy kit (Quiagen Inc., Valencia, CA), following the manufacturer's instructions. We used the mismatch amplification mutation assay (MAMA) described by Cha *et al* [29] with some modifications. The MAMA is specific for the codon 12 GGA to GAA mutation in the Ha-*ras*-1 gene. Briefly, this method uses two sets of primers: one targets the mutation and the other a control area in the genomic DNA. The mutant specific mismatch primer PAA (5'-CTTGTGGTGGTGGGCGCTGAA-3'), the Pmnl2 (5'-ACTCGTCCACAAAATGGTTC-3') and the control primers (P1: 5'-

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CCTGGTTTGGCAACCCCTGT-3' and Pmnl2: 5'-ACTCGTCCACAAAATGGTTC-3')

were used at a 40 ng/μl concentration. The PCR was performed using Platinum Supermix

(Invitrogen, Carlsbad, CA). The PCR products were run in a 2% agarose gel (Gibco). The

expected size of the non-mutated Ha-*ras*-1 gene is 128 bp while the mutated Ha-*ras*-1

gene is 74 bp.

Statistics

Statistical significance of the incidence of neoplastic lesions was determined using the X^2

Test.

Results

Normal ducts developed from tumor cells.

The transplantation of mammary tumor cells into CFPs gave rise to ductal outgrowths

that were phenotypically normal at the time of harvesting (6 months after injection of

tumor cells). Normal ductal development was observed in almost all animals, regardless

of the host's age at transplant or parity status. Ductal outgrowths were not observed in the

mammary glands of animals that developed large tumors, as the tumors encompassed the

entire fat pad at the time of tissue collection. From these data, we cannot rule out the

possibility that ductal growth occurred.

Secondary tumor development inversely correlated with the age of the host.

The transplanted donor tumor cells gave rise to a variety of outgrowths, ranging from

large secondary tumors to microscopic neoplastic lesions as well as normal ductal

development. The tumor incidence correlated inversely with the age of the stroma. That is, the highest tumor incidence was observed in the younger animals: 75% of the 24-day-old hosts and 100% of the 52-day-old hosts developed secondary tumors (Table 1, Fig. 1). The incidence of secondary tumors decreased to 50% in the 80-day-old and to 18.2% in the 150-day-old hosts. The twice-parous group only developed phenotypically normal ducts; no tumors or microscopic neoplastic lesions were observed in this group. Statistically significant differences were observed between the 52-day-old group and the parous ($p=0.001$), the 150- ($p=0.001$) and the 80-day-old ($p=0.029$) groups. The 24-day-old group was different from the parous ($p=0.016$) and the 150-day-old ($p=0.022$) groups (Table 1).

We performed histopathological analyses of donor and secondary tumors as well as the microscopic neoplastic lesions following the criteria described by Russo *et al* [30]. The donor tumors were carcinomas, papillary and cribriform types; the secondary tumors were classified mostly as infiltrating carcinomas, cribriform and comedo types. Figure 2 shows an example of a donor tumor and the outcome of the transplantation of its neoplastic cells into a 24-, 52- and 80-day-old host. As mentioned above, tumors developed only in the younger animals.

Mutated Ha-*ras*-1 gene expression is seen in secondary tumors and ducts.

In order to recognize the tumor cells that were injected into the host's CFPs, we used the codon 12 GGA to GAA mutation in Ha-*ras*-1 gene as a marker of tumor origin. This marker was chosen because it has been claimed that NMU induces this particular point

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mutation in the Ha-*ras*-1 gene of mammary epithelial cells [31]. All the donor tumors carried the codon 12 mutation and the same mutation was observed in both types of secondary outcomes, namely, tumors or normal ductal development, a confirmation of their tumor origin (Fig. 3).

Discussion

The data collected suggest that an inoculum of just 5×10^4 neoplastic epithelial cells transplanted into the mammary stroma of syngeneic hosts resulted in tumor takes as well as normal ducts. This is consistent with Rivera *et al.*'s observations [15;25]. Significantly, we also uncovered that the neoplastic outcome depended on the age of the host and/or their parity status at the time the epithelial cells were inoculated.

The development of the mammary gland is regulated by hormonal cues triggered by puberty and pregnancy. These cues orchestrate stromal-epithelial interactions leading to ductal growth, invasion, lateral branching and alveolar development [32]. In our experiments, the time points for donor tumor cell and stroma recombination were chosen to represent particular developmental stages of the normal mammary gland. *A priori,* we assumed that the CFP underwent developmental changes similar to those observed in the intact mammary gland. We based this assumption on the fact that both the stroma and the epithelium respond to ovarian hormones during the postnatal development of the mammary gland. Furthermore, some aspects of epithelial development are influenced by signals initiated in the stroma. For instance, Cunha *et al.* observed that mammary ductal

growth and branching during puberty are dependent upon estradiol signaling through the estrogen receptor-α present in the stroma cells [33].

We chose two time points during which ductal invasion of the stroma takes place in the intact gland, namely 24 days of age (the beginning of ductal invasion) and 52 days of age (when evident ductal growth is underway). This latter age also represents the well-known window of maximal vulnerability to chemical carcinogens in tumor-susceptible strains of rats [34;35]. The other time points were 80 days of age, when the ducts reach the edge of the fat pad, and 150 days of age, when the mammary gland of a virgin animal is considered an organ where no major tissue remodeling is observed [36;37]. We also took into account the fact that there is an inverse correlation between mammary tumor incidence and the age at which the carcinogen is administered [28;34;38;39]. We observed that the CFPs of younger animals (24-52 days of age) allowed for maximal secondary tumor development as well as ductal growth, whereas aged stroma (80-150 days of age) shifted the outcome towards normal ductal growth and a lower incidence of secondary tumors. In other words, we verified an inverse correlation between age and the detection of neoplasms that parallels the relationship between age and susceptibility to carcinogens in the mammary gland.

The mammary stroma undergoes biochemical and cellular changes associated with the endocrine milieu. The extracellular matrix (ECM) components of rat mammary gland stroma are modified by the animal's reproductive state [40]. More recently, Schedin *et al.* observed that the mammary matrix isolated from parous rats loses the ability to promote

complex glandular development when compared to the matrix isolated from nulliparous mammary glands [41]. Non-carcinogenic mouse mammary epithelial FSK-3 cells grown in a 3-dimensional culture formed duct-like structures that invaded the substratum when cultured onto matrix from nulliparous 52-day-old rats. In contrast, the presence of matrix from parous rats restricted the formation of complex structures [41]. Herein, we observed that the stroma of parous rats not only restricted the development of a secondary tumor but, more importantly, instructed the neoplastic epithelial cells to form normal ductal outgrowths. Both Schedin *et al.*'s and our study strongly suggest that cellular and extracellular components of the stroma contribute to the protective effect of pregnancy against tumor formation. In addition, the stroma also plays a main role in the reversal of the neoplastic phenotype (Table 1, Fig. 2). Moreover, the results presented herein suggest that the development of a protective effect against tumor formation observed in these animals does not require the contribution of the epithelial compartment, since the ductal epithelium was removed from the mammary gland at 10 days of age. It appears premature at this time to suggest which of the numerous cellular and extracellular stroma components play a definitive role in either the carcinogenic process or in its reversion.

Can these results in rodent mammary glands be extrapolated to clinical and epidemiological data in human breast cancer? The long-term outcome of survivors of the 1945 Hiroshima and Nagasaki nuclear explosions represents a relevant subject for comparison. The dose-specific excess relative risk for breast cancer increased 13-fold in women exposed before age 20 who went on to develop clinical cancer decades later [42], whereas this risk was significantly lower in older women. This suggests that

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susceptibility to radiation decreases with age. Epidemiological data also show that the frequency of *in situ* breast carcinoma is higher in middle-aged women compared to the frequency of invasive carcinoma found in the elderly [43;44]. This pattern, in which the presence of ductal carcinoma *in situ* alone or associated with invasive carcinoma decreases with age, was reported in a more recent study by Wazer *et al* [45]. It has been proposed that this lack of correlation between age and incidence is compatible with spontaneous regression of subclinical lesions [23].

Finally, these experiments add to the mounting evidence that the stroma plays a crucial role in carcinogenesis and reversion. The precise role of its diverse components deserves to be explored aggressively.

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Figure Legends

Figure 1: The incidence of secondary tumors decreases with the age of the stroma. The parous host only developed normal ductal outgrowths. *Statistically different from twice-parous, 150- and 80-day-old host groups. ** Statistically different from twice-parous, and 150-day-old host groups.

Figure 2: Diverse results were obtained from the same tumor donor. (A) Papillary carcinoma used as a donor tumor. (B) Secondary tumor developed in a 24-day-old host. (C) Secondary tumor developed in a 52-day-old host. (D) Normal ductal outgrowth developed in an 80-day-old host. In both secondary tumors there is a noticeable increase in the deposition of extracellular matrix and the number of glands is reduced, showing a less differentiated phenotype. Scale bar: 100 μm in A, B and C; 2 mm in D.

Figure 3: Examples of Ha-*ras*-1 expression in donor tumors and their outcomes. Each number represents one sample and its Ha-*ras*-1 expression: the left lane is the endogenous Ha-*ras*-1 and the right lane is the mutated gene. Samples 1, 3, and 5 are examples of donor tumors. Sample 2: DNA was extracted from a normal ductal outgrowth developed in an 80-day-old host injected with neoplastic cells from Sample 1. Sample 4: DNA was extracted from a secondary tumor developed after the transplant of cells from Sample 3. Samples 6 and 7: DNA was extracted from a normal ductal outgrowth and a secondary tumor developed in 80- and 24-day-old hosts, respectively. Both hosts were inoculated with Sample 5. All donor tumors carry the codon 12 GGA to

GAA mutation and the same mutation can be seen in both types of secondary outcomes,

i.e. tumors or normal ductal development.

Table 1: Outcome of neoplastic epithelial cell injection into hosts at different ages and parity status

Host's age	Initial n	Final n	Tumors	Outgrowths
Twice-Parous	7	5	0/5 (0%)	5/5 (100%)
150 day-old	11	11	2/11 (18.2%)	11/11 (100%)
80 day-old	11	10	5/10 (50%)	7/10 (70%)
52 day-old	10	8	8/8 (100%) *	7/8 (87.5%)
24 day-old	9	8	6/8 (75%) **	5/8 (62.5%)

* Statistically different from twice-parous, 150- and 80-day-old host groups.

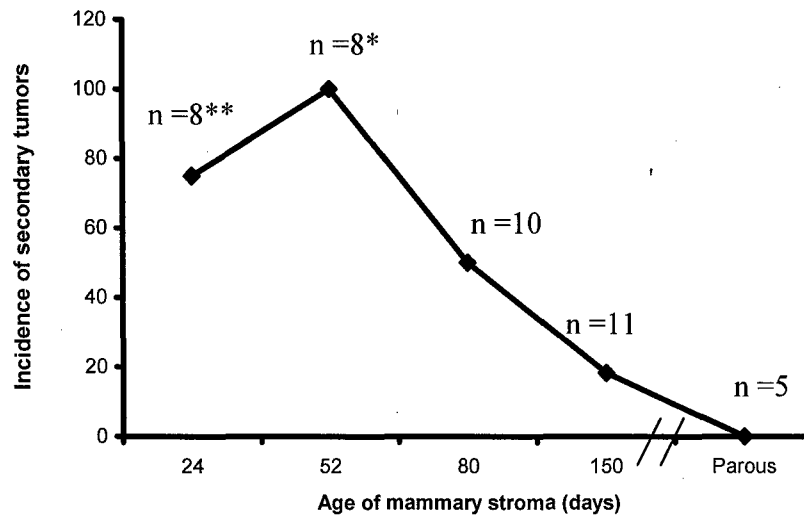** Statistically different from twice-parous, and 150-day-old host groups.

Figure 1

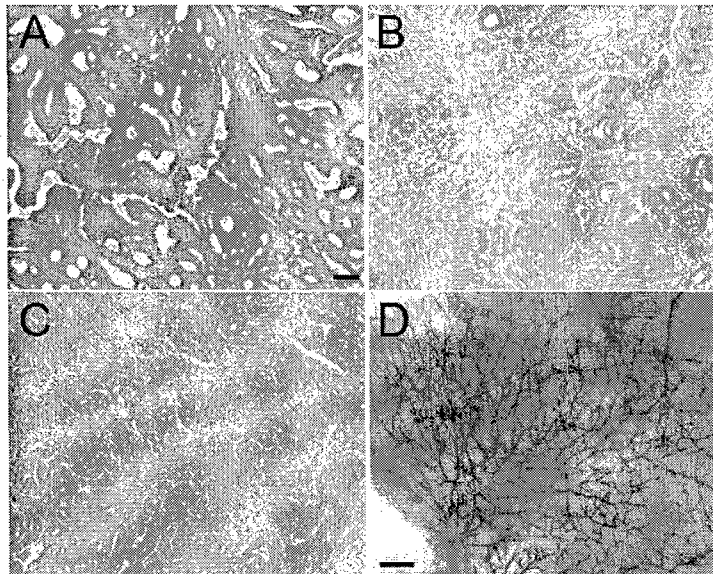**Figure 2**

Figure 3

128 bp →
74 bp →